BLUBBER AND BEYOND: THE ROLE OF LIPIDS IN THERMOREGULATION AND ENERGY RESERVES OF PHOCID SEALS

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Abstract

Phocid seals rely on lipids in the form of a blubber layer as insulation and lipids as energy sources in blubber and muscle. The amount and lipid composition of blubber and other lipid stores vary throughout life within and among species of phocid seals. I hypothesized that this variation in regulation, allocation, and interactions among lipid stores is influenced by species, ontogeny, and tissue-specific thermal regimes in polar phocids: harp (*Pagophilus groenlandicus*), hooded (*Cystophora cristata*), and Weddell (*Leptonychotes weddellii*) seals.

I investigated the thermoregulatory strategy of neonatal harp, hooded, and Weddell seals, and throughout the transition to an aquatic environment in harp seals. All three species had similar thermal resistance, though it was achieved differently using either lanugo or blubber. While there was variation in the main thermoregulatory strategy among species, no species possessed all thermal adaptations of adults. Harp and Weddell seals had higher surface area to volume ratios (SA:V), thus higher potential heat loss, though compensatory mechanisms for heat production were different between species. Harp seals were the only species with the potential for nonshivering thermogenesis (NST) in brown adipose tissue (BAT), whereas Weddell seals had the highest potential for shivering thermogenesis (ST) in their skeletal muscle. Hooded seals relied on blubber, and had a significantly lower SA:V than the other two species. As harp seal pups develop, their potential for NST declines and they shift to a reliance on blubber for insulation. By late weaning, harp seal pups have similar insulative capabilities as adults, and can likely meet the thermoregulatory challenges associated with living in water. In neonatal and young seals that have little blubber, other lipid stores such as BAT and skeletal muscle lipids provide heat-generating mechanisms (NST or ST) to offset potentially high rates of heat loss. The potential for NST declines with age, as the blubber layer develops in harp seals, and weaned pups look to have similar insulative capabilities as adults.

While phocid adults rely on blubber for insulation and maintain a thermal gradient across the tissue, otariids (fur seals and sea lions) instead maintain an external gradient across a thick fur layer. This has implications for the underlying lipid composition of blubber, as the fatty acids (FA) that make up this lipid respond differently to temperature. In phocid blubber, latitude (a proxy for environmental temperature) had a positive correlation with the proportion of polyunsaturated fatty acids, but a negative correlation with saturated fatty acids. In otariids, these

trends were reversed. This suggests interactions between blubber and the ambient environment play a role in the overall relative proportions of FA classes in blubber. Unlike in blubber, the FA class composition of harp, hooded, and Weddell seal skeletal muscle was similar among species. In adult female Weddell seals, the relative proportions of individual FA in blubber and muscle were significantly different between tissues; these differences persisted across seasons, and were independent of female reproductive state. It appears that the FA in blubber and muscle reflect the tissues' roles within the body. Blubber contained a greater proportion of monounsaturated FA, which remain fluid at lower temperatures, while the muscle contains a larger proportion of SFA, which produce the greatest amounts of ATP per mole oxidized to support metabolism. In adult phocids, the FA composition of the blubber layer appears to be influenced by environmental interactions with latitude (temperature), in accordance with the location of and steepness of the thermal gradient through the blubber layer. Finally, environment looks to have little influence on the FA stores in skeletal muscle, and seasonally persistent tissue differences between blubber and muscle highlight how lipid is dynamically modulated within the body of phocid seals. How and what lipids are allocated to blubber is a mixture of abiotic and biotic cues throughout life, linked to thermodynamics, ambient environment, and energy dynamics.

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General Introduction

In marine mammals, as in all mammals, when energy intake is greater than energy demand, excess energy is stored. Lipids made of triacylglycerols (TAG) are the predominant storage molecules because they are hydrophobic. Therefore, TAG are stored with less water (~10% water) than carbohydrates or proteins (~ 30% water), making them lighter per unit energy. Lipid yields 0.47 mol ATP g^{-1} of lipid, or 423 ATP mol^{-1} versus 0.20 mol ATP g^{-1}, or 34 ATP mol^{-1} carbohydrate (Pond 1998; Gurr et al. 2002). TAG consist of a glycerol backbone bound by ester bonds to three hydrocarbon fatty acid (FA) chains (Pond 1998; Gurr et al. 2002). In the case of negative energy balance, FA are hydrolyzed from their glycerol backbone by lipases. The FA are transported through the bloodstream bound to albumin, and transported across cell membranes by a suite of tissue-dependent translocase proteins, and oxidized to substrates that contribute to the formation of ATP. The ability to stockpile large amounts of energy in adipose deposits has allowed animals to survive temporally and spatially distant from food sources, and inhabit areas previously inaccessible because of environmental conditions (Young 1976; Pond 1992; Fruhbeck et al. 2001; Gurr et al. 2002).

Lipid stores

In mammals, lipids are stored primarily in 12 white adipose tissue (WAT) deposits located throughout the body. Adipocytes are simple in structure, with one lipid droplet per cell (unilocular), few mitochondria, and a supportive collagen framework (Pond 1998). The amount of lipid stored as WAT is generally indicative of an individual's overall condition and nutritional status (Pond 1978; Hanks 1981; Weber 2011; Champagne et al. 2012). WAT itself also plays an important role as an endocrine organ regulating the uptake, storage, and controlled release of lipids through the signaling actions of adipokines such as leptin, hormones such as adiponectin, and inflammatory factors such as cytokines (Mohamed-Ali et al. 1998; Trayhurn et al. 2006). In marine mammals, the main lipid storage site is a large subcutaneous deposit referred to as the blubber layer. Blubber has many other roles in the body including insulation because the low conductivity of lipid slows heat loss (Worthy and Edwards 1990; Kvadsheim et al. 1996; Rosen and Renouf 1997; Kvadsheim and Aarseth 2002; Dunkin et al. 2005; Liwanag et al. 2012b).

While WAT is the main site of lipid storage in the body, lipid is also stored in other tissues. For example, many mammalian species possess a more specialized thermogenic adipose tissue known as brown adipose tissue (BAT) (Afzelius 1970; Cannon and Nedergaard 2004). Unlike white adipocytes, brown adipocytes are multilocular, have high mitochondrial densities, and are highly vascularized and innervated (Trayhurn 1995). BAT stores large amounts of TAG and metabolizes it at high rates to fuel nonshivering thermogenesis (NST). The thermogenic capacity of BAT originates from the expression of uncoupling protein 1 (UCP1) in the mitochondria (Cannon et al. 1982; Golozoubova et al. 2006), which uncouples oxidative phosphorylation and ATP synthase. Cold-induced stimulation of UCP1 occurs through the sympathetic nervous system via norepinephrine and β_3-andrenergic receptors (Carneheim et al. 1984; Trayhurn 1995). Chronic cold stimulation results in adaptive thermogenesis in BAT by increasing the capacity for NST by increasing mitochondrial density, β-oxidation rates, tricarboxylic cycle (TCA) enzyme activities, and expression of UCP1 (Cannon and Nedergaard 2004; Cannon and Nedergaard 2011). Accordingly, the thermogenic capacity of BAT is determined by the amount of BAT, UCP1 expression, enzyme activity, and mitochondrial density (Trayhurn 1995; Cannon and Nedergaard 2011). Among mammals, the presence and functionality of BAT varies with ontogeny and species. For example, precocial neonates, such as guinea pigs (*Cavia porcellus*) and reindeer (*Rangifer tarandus*), are born with high thermogenic capacity BAT (Cannon and Nedergaard 1985; Soppela 2000). In contrast, in altricial species, such as hamsters (*Mesocricetus auratus*), the thermogenic capacity of BAT develops over several days or weeks (Cannon and Nedergaard 1985; Soppela 2000). A few species, such as domestic pigs (*Sus scrofa domesticus*) do not possess UCP1 or BAT (Trayhurn et al. 1989) and rely solely on other thermogenic mechanisms such as shivering.

Triacylglycerols are stored as lipid droplets in skeletal muscle fibers in addition to WAT and BAT. These intramuscular lipid droplets typically account for 0.5% of skeletal muscle volume in healthy (height weight proportionate) humans, up to 3.5% in obese individuals (Goodpaster et al. 2000), but up to 15% in some marine mammals (Trumble et al. 2010). Muscle is the largest metabolic consumer of lipids (Dyck et al. 1997; Watt and Hoy 2012), relying on both intramuscular lipid and lipids transported from WAT to fuel both shivering and controlled muscle contraction. Intramuscular lipids are immediately available to mitochondria for oxidation to ATP. Enzymes in the β-oxidation pathway break down FA chains into acetyl-CoA, which then

enters the TCA cycle (Gurr et al. 2002). Lipid droplets are often closely associated with mitochondria, likely to reduce substrate transport distances (Dyck et al. 1997; Guglielmo 2010; Price 2010; Weber 2011; Watt and Hoy 2012).

Regulation of amounts and types of lipid in the body

While diet is the main source and a large determinant of the types and amounts of FA present in TAG stores (Raclot and Groscolas 1993; Iverson et al. 2004; Budge et al. 2006), ingested FA can be modified by elongation, shortening, or desaturation (Gurr et al. 2002). In addition, endogenous *de novo* synthesis of certain FA affect the overall FA composition of lipid stores (Budge et al. 2004; 2006; Guglielmo 2010). Fatty acids stored by upper trophic level predators generally have 12 to 24 carbons and fewer than six double bonds (Dalsgaard et al. 2003). Short- and medium-chain FA are simply transported to, and oxidized by, the liver (Budge et al. 2006). Long-chain FA are hydrolyzed, absorbed across the intestinal mucosa, reformed into acyl lipids, and then transported via circulating chylomicrons to tissues for storage (WAT), oxidation (muscle), or modification (liver).

The physiochemical properties of FA are influenced by the degree of saturation and chain length (Raclot and Groscolas 1993; Raclot and Groscolas 1995; Connor et al. 1996; Vaillancourt and Weber 2007; Vaillancourt et al. 2009). The number of double bonds determines thermal stability among three major classes of FA. Saturated fatty acids (SFA) have no double bonds between carbon molecules, monounsaturated fatty acids (MUFA) contain one double bond, and polyunsaturated fatty acids (PUFA) contain multiple double bonds. SFA generate the largest amount of ATP mol^{-1} of FA oxidized, produce the lowest amount of ATP mol^{-1} of oxygen consumed (Gurr et al. 2002; Trumble and Kanatous 2012), have the highest melting point, and are solid at typical mammalian body temperatures (~ 37 °C) (Gurr et al. 2002). MUFA are intermediate between SFA and PUFA in terms of energy, oxygen use, and melting point (~14°C) (Gurr et al. 2002). Fully metabolizing long-chain (> 18 carbons) PUFA through β-oxidation uses less oxygen than MUFA or SFA, but because of the energy required to break the double bonds, it provides less ATP per $mole^{-1}$ FA (Gurr et al. 2002; Trumble and Kanatous 2012). Few PUFA can be synthesized in mammals, and those designated omega-3 and omega-6 are only available from dietary sources (Holman 1958). Additionally, PUFA have the lowest melting point (-50 – -70°C), and are liquid at mammalian body temperatures (Gurr et al. 2002).

The mobilization of FA for oxidation follows a general pattern based on the degree of unsaturation and chain length. For a given number of double bonds, shorter chain FA are mobilized first; but for a given chain length, FA with a higher degree of unsaturation are mobilized first (Raclot and Groscolas 1993; 1995; Raclot 2003). Mobilization rates are unaffected by the relative amount of each FA, until the supply of a particular FA is depleted (Ogawa et al. 1992; Raclot and Groscolas 1995; Kuroshima et al. 1995; Groscolas and Herzberg 1997; Raclot 2003). Neither fasting or cold exposure change the pattern of mobilization (Raclot and Groscolas 1995; Vaillancourt and Weber 2007), though there are exceptions with respect to FA classes that have direct effects on thermoregulation and metabolism. For example, many species such as reindeer (Soppela 2000) preferentially alter the FA composition of tissues regularly exposed to low temperatures to maintain the fluidity of the membranes (homeoviscous adaptation) (Sinensky 1974). In whales, the blubber is stratified so that FA important in maintaining structure and flexibility of the blubber (MUFA, PUFA, and in the case of certain whales, wax esters) are located nearest the skin and body surface (Strandberg et al. 2008), where they remain fluid when exposed to low ambient temperatures. Energetic demands may also influence FA profiles (Price 2010; Price et al. 2013). For example, in migratory birds, long-chain PUFA are preferentially oxidized by skeletal muscle, which results in lower overall energy and oxygen use during migration (McWilliams et al. 2004; Pierce and McWilliams 2005), although the specific mechanism for selective use is still debated (for review see Price 2010). In contrast, ground squirrels (*Ictidomys tridecemlineatus*) preferentially save PUFA during torpor and hibernation to maintain the fluidity of tissues and membranes when core body temperature drops (Price et al. 2013). Therefore, the FA profiles (i.e., the proportions of individual fatty acids) of the three major lipid stores in the body (WAT, BAT, muscle) are likely tailored to match the needs of the tissue as these roles differ (Iverson et al. 1992; Raclot 2003; Budge et al. 2004; Iverson et al. 2004; Rosen and Tollit 2012). Additionally, general lipid availability, amount, and use likely varies with age, season, and diet. For example, during periods of low calorie intake, or when fat contributes 10% or less of the total caloric intake, FA synthesis, and modification of SFA and MUFA increases to meet the tissue-specific demands (Iverson et al. 2004; Budge et al. 2006). During periods of negative energy balance and fasting, modifications cease, and FA are mobilized at a greater rate to meet metabolic demands (Iverson et al. 1992; Raclot and Groscolas 1993; Budge et al. 2004; Iverson et al. 2004)

Lipids in phocids

Adult phocids have developed a suite of morphological and physiological adaptations to counteract elevated heat transfer when submersed in water (Irving and Hart 1957; Scholander et al. 1950). Heat loss is typically reduced through a combination of lower surface area to volume ratios (SA:V) as compared with terrestrial animals of similar size (Innes et al. 1990; Oftedal et al. 1991), low peripheral blood flow (vasoconstriction), counter-current heat exchangers (Scholander et al., 1950), and an insulating layer of subcutaneous blubber (Scholander et al. 1950; Dunkin et al. 2005; Liwanag et al. 2012a; Liwanag et al. 2012b). As in other endotherms, phocids can increase heat production by raising their metabolic rate and/or initiating shivering thermogenesis, although these are metabolically costly (Worthy and Lavigne 1987; Hindle et al. 2006; Yeates et al. 2007; Liwanag et al. 2009; Cannon and Nedergaard 2011; Humphries and Careau 2011). In general, these adaptations provide adult phocids with broad thermal neutral zones, that extend beyond the average ambient temperatures of their habitat (Hokkanen 1990).

Most phocid pups have little or no blubber and rely instead on a lanugo coat for insulation (Scholander et al. 1950; Ling 1974; Elsner et al. 1977; Oftedal et al. 1991; Kvadsheim and Aarseth 2002). In addition, young phocids have a large SA:V (Blix and Steen 1979; Oftedal et al. 1991) and poorly developed vasocontrol (Lapierre et al. 2004) compared with adults. Yet they maintain a stable core body temperature (Scholander et al. 1950; Irving and Hart 1957; Blix and Steen 1979; Little 1995), even when ambient temperatures are well below freezing (Ortisland and Ronald 1978; Blix and Steen 1979). To maintain euthermia, young seals may employ thermoregulatory strategies not used by adults, such as shivering or nonshivering thermogenesis, to increase their thermogenic capacity. Thermoregulatory strategies may vary in species with different early developmental strategies, and may shift as pups nurse, develop a blubber layer in preparation for life in the water. Previous studies on thermoregulation in young phocids have primarily focused on insulation or mechanisms for the prevention of heat loss (Scholander et al. 1950; Blix and Steen 1979; Kvadsheim and Aarseth 2002). Little work quantifying heat-generating mechanisms has been conducted. It is often assumed species born with a thin blubber layer are better insulated, and species with lanugo rely on NST (Irving and Hart 1957; Blix and Steen 1979; Noren et al. 2008). Few studies have quantified a

comprehensive suite of thermoregulatory mechanisms in a given species, age class, or across early development.

When phocids reach adulthood, the blubber layer stores up to 75% of their total body lipids (Ryg et al. 1988; Reilly and Fedak 1990; Ryg et al. 1993; Liwanag et al. 2012b). It evolved in response to thermoregulatory challenges imposed by the aquatic lifestyle of marine mammals, as the higher conductivity of water compared with air results in elevated rates of heat loss (Scholander et al. 1950; Irving 1973; Ryg et al. 1993; Liwanag et al. 2012b). Because blubber is relatively incompressible and has low conductivity (0.18 – 0.20 W m^{-1} $°C^{-1}$; Blix et al., 1979; Kvadsheim and Aarseth, 2002; Liwanag et al., 2012b), the evolution of blubber as internal insulation allows marine mammals to maintain a large thermal gradient between their core and body surface temperatures, thus reducing heat loss (Ortisland and Ronald 1978; Ryg et al. 1988; Ryg et al. 1993; Kvadsheim and Aarseth 2002; Liwanag et al. 2012b). Additionally, a large blubber layer adds to body volume, which helps reduce the SA:V, decreasing the surface over which heat transfer to the environment can occur (Innes et al. 1990). Therefore, adult phocids rely on a blubber layer for insulation, whereas their thin fur layer offers little thermal protection (Kvadsheim and Aarseth, 2002; Liwanag et al., 2012a,b).

In addition to serving a thermoregulatory role, the blubber layer is also critical in maintaining energy balance. It serves as the main energy reserve and source for TAG, and provides lipids for milk production during lactation (Champagne et al. 2012; Arriola et al. 2013; Fowler et al. 2014). However, the role as an energy reserve can conflict with the role in thermoregulation because the total insulation provided by blubber is proportional to its thickness (Worthy and Edwards 1990; Rosen and Renouf 1997; Kvadsheim and Aarseth 2002; Liwanag et al. 2012b). Theoretically, if too many TAG from blubber are used to fuel metabolism, then the blubber may become too thin for phocids to maintain thermal homeostasis in the water (Ryg et al. 1988; Rosen and Renouf 1997; Rutishauser et al. 2004). The dual function of blubber as an energy source to be used and a thermal barrier to be conserved, means that the thickness and FA composition of the blubber layer should be carefully regulated (Nordøy and Blix 1985; Worthy and Lavigne 1987; Ryg et al. 1988; Rosen and Renouf 1997; Rutishauser et al. 2004; Noren and Wells 2009; Liwanag et al. 2012b).

There is variation in the role of the blubber layer within the pinniped clade. Unlike phocids, the extent to which blubber serves as a thermal barrier differs among the otariids (a

polyphyletic family) (Gentry 1973). In fur seals, the blubber layer is primarily used for energy storage, as their thick fur coat keeps skin and underlying tissues warm (Gentry, 1973; Liwanag et al. 2012a). In contrast, sea lions rely on a combination of blubber and fur for thermoregulation (Gentry 1973; Liwanag et al. 2012a). The variation in the importance of blubber in thermoregulation among adult pinnipeds suggests differences in the FA profiles of the blubber could occur between these families. Further, blubber often has high levels of unsaturated FA (UFA), because UFA have a lower melting point than SFA (Irving et al. 1957). Thus, for individuals and species that maintain a thermal gradient across the blubber, this tissue may contain higher relative proportions of UFA to maintain flexibility when the blubber layer is cool (Irving et al. 1957; Sokolov 1962; Trumble et al. 2010; Fowler et al. 2014). Under this scenario, species that live in colder environments and/or maintain a thermal gradient across internal blubber rather than external fur might have greater proportions of UFA than those in warmer environments. In addition, because a thinner blubber layer provides less resistance to heat flux, individuals / species with thinner blubber insulation may also have more MUFA and PUFA in the blubber. Larger animals have a lower SA:V (Innes et al. 1990) and can carry a thicker blubber layer compared with smaller animals (Ryg et al. 1993). Thus, body size (mass) and condition (blubber depth or total body lipid) may also influence the relative proportions of FA in blubber with lighter animals or those with thinner blubber having a greater relative proportion of UFA in their blubber than heavier animals.

Regulation of the allocation of lipid resources and fate of specific FA in the body is not well understood in phocids. In addition to the blubber layer, phocids store large amounts of lipid as intramuscular lipid droplets (Trumble et al. 2010), which fuel aerobic metabolism while diving (Reed et al. 1994; Kanatous et al. 1999; Kanatous et al. 2008). Muscle temperature is kept close to core body temperature, and does not exhibit the thermal gradients present in the blubber layer (Ponganis et al. 1993; Noren et al. 2008). Because there is little thermal constraint in the muscle, muscle may contain greater proportions of high energy FA and fewer low-melting point FA. The main locomotory muscle, the *Longissimus dorsi,* consists primarily of oxidative (Type I and IID) fibers (Kanatous et al. 1999; Kanatous et al. 2002; Watson 2003; Moore et al. 2014), suggesting that intramyofibril TAG are an important fuel source for locomotory activity. The same underlying metabolic pathways govern muscle metabolism regardless of species. Accordingly, the relative proportion of FA classes in the muscle should be similar across species

in comparable body condition and at the same point in their annual cycle, regardless of differences in diet. The greatest ATP demands for phocids occur while underwater (Kooyman et al. 1981), with limited oxygen, and limited resupply of FA because of vasoconstriction. Because of this, stored intramuscular FA may be those that produce the most ATP $mole^{-1}$ FA (SFA) or those that use little oxygen per ATP produced (PUFA) (Trumble and Kanatous 2012). Thus, the FA profile in muscle may differ from that of blubber, where incorporation of specific FA is likely influenced by thermoregulatory demands and the need to maintain fluidity at low temperatures.

Phocids experience seasonal changes in body condition (e.g., total body lipid, blubber depth) in response to reductions in diving and foraging effort during the reproduction and molting periods (Stirling 1969; Castellini et al. 1985; Boyd et al. 1993; Arnbom et al. 1993; Bennett et al. 2001; Sparling et al. 2006; Bennett et al. 2007). At the end of reproduction and molting, animals typically possess their lowest energy reserves and thinnest blubber layers. Over the winter foraging period, seals regain lipid in both muscle and blubber. These seasonal shifts in energy balance likely influence the FA profile of both the muscle and the blubber as energy balance influences the storage, use, and synthesis of FA.

In this study, I investigated the role of lipids in thermoregulation in blubber and metabolism in skeletal muscle of phocid seals. The aim of the present study was to better understand the allocation and interactions among lipid stores at different times of life in highly lipid dependent species. I targeted three species of phocids, harp (*Pagophilus groenlandicus*), hooded (*Cystophora cristata*), and Weddell (*Leptonychotes weddellii*) seals. All three species live in polar environments, where both ambient and water temperatures remain low year round, and animals haul out on ice to give birth and to molt. Using a comparative approach of species that live in similar environments helps to control for potentially confounding variables, so that underlying physiological mechanisms may emerge. I hypothesized that major shifts in age and energy allotment would influence the distribution and use of lipids in the body. Further, I hypothesized that other lipid tissues in the body, such as BAT and skeletal muscle, would be key to thermoregulation, especially in species/age classes that lack developed blubber. To broaden the understanding of how biotic and abiotic factors influence blubber composition of adult pinnipeds, I use a mix of direct measurements of body condition, blubber FA, latitude, and

literature data from a variety of phocid and otariid species. I hypothesize FA in the blubber will vary in predictable ways in response to environment and/or physiological constraints.

Study species

Harp, hooded and Weddell seals are representative members of three different branch points on the phylogenetic tree of phocids (Higdon et al. 2007; Berta and Churchill 2012). They also represent a broad spectrum of the ecology of phocid pups and adults, varying in ontogeny, body size, dive behavior, and diet. Large aggregations of individuals during the breeding season make them accessible for study, and specific life stages can be targeted for sampling. Harp seals are born on pack ice in the Gulf of St. Lawrence, Canada, and along the east coast of Greenland in March and early April (Stewart and Lavigne 1980). Yet, harp seal pups have a small body size (~10 kg or ~ 7% of maternal mass, Anderson and Fedak 1987), a very thin blubber layer, and a wettable lanugo coat for insulation (Kvadsheim and Aarseth, 2002). Previous research has suggested harp seal pups rely on BAT while nursing (Grav et al. 1974; Grav and Blix 1976), though the presence of UCP1 has not been confirmed. Pups nurse for 12 days, gaining an average of 2.3 kg day^{-1} (Kovacs and Lavigne 1985) before they are weaned, and then pups fast for 4–6 weeks before beginning to forage independently (Stewart and Lavigne 1980). Hooded seals are born in a similar location as harp seals, and the herds often overlap (Stewart and Lavigne 1980). Despite similar birth environments, early ontogeny of hooded seal pups is a stark contrast to harp seal pups. Hooded seal pups are larger (~25 kg or typically 11% of maternal body mass; Bowen et al., 1985; Anderson and Fedak, 1987), they molt their lanugo pelage *in utero* and are born with adult-like pelage, but have a blubber layer that provides insulation (Bowen et al. 1985; Oftedal et al. 1991; Lydersen et al. 1997). Hooded seals nurse for four days, gain 7.6 kg day^{-1} while nursing (Bowen et al. 1985; Bowen et al. 1987) and after weaning, remain hauled out for approximately four weeks during the post-weaning fast (Bowen et al. 1985; Bowen et al. 1987). At the opposite pole, the larger Weddell seals are born on stable fast ice in October and November (austral spring) around Antarctica (Elsner et al. 1977). Though similar in absolute mass to hooded seal pups, Weddell seal pups are small relative to maternal mass (~30 kg or ~ 6%, Anderson and Fedak 1987; Wheatley et al. 2006), and they are born with little blubber and a wettable lanugo (Elsner et al. 1977). Pups nurse for 32–45 days, and gain an average of 2 kg day^{-1} while nursing. Unlike either harp or hooded seals, Weddell seal pups enter

the water at approximately two weeks of age, and begin swimming with their mother during the nursing period (Ray and Smith 1968; Hill 1987).

Adult harp seals congregate in pack-ice flows (whelping patches) to give birth, nurse and mate (Stewart and Lavigne 1980). Adults typically weigh 113.4 ± 15.1 kg (reproductive), have an average dive duration of 5–10 minutes, are capable of dives > 500 m, though average depth is 100–200 m, and feed on capelin (*Mallotus villosus*) and pelagic amphipods (*Themisto* spp.) (Folkow et al. 2004; Haug et al. 2004). When not aggregated for breeding, individuals are dispersed, and less accessible for study. Breeding adult hooded seals overlap spatially and temporally with breeding harp seals. Adult hooded seals females weigh 232.6 ± 71.1 kg (reproductive; Lavigne and Kovacs 1988). Adults are capable of dives up to 1016 m, though average dives are 100–600 m and 5–25 min long (Folkow and Blix 1999). Summer diet consists of squid (*Gonatus fabricii*) and Arctic cod (*Boreogadus saida*) (Haug et al. 2004; Haug et al. 2007), whereas winter diet consists of squid and capelin (Haug et al. 2004). Both harp and hooded seals are less accessible for study when not aggregated for breeding, as individuals are dispersed during the molt. Adult Weddell seals give birth, nurse, and mate during the austral spring (October/November) followed by the molt in fall (January/February) (Lugg 1966; Stirling 1969; Fenwick 1973). After the end of the molt, adults spend the overwinter period foraging throughout the Ross Sea to regain lipid reserves lost before returning to McMurdo Sound the following spring (Lugg 1966; Stirling 1969; Fenwick 1973). Adult female Weddell seals weigh 320.7 ± 10.3 kg in fall (post–molt), and 335.8 ± 14.2 kg (non-reproductive) to 413.7 ± 13.3 kg (reproductive) in spring (Shero et al. 2014). Dive duration averages ~ 20 min to mean depths of 150 m (Castellini et al. 1992), though they are capable of diving for > 90 min, and to depths of ~ 1200 m (Goetz unpublished data). Their diet consists mainly of Antarctic silverfish (*Pleuragramma antarcticum*), Antarctic toothfish (*Dissostichus mawsoni*), and cephalopods (Burns et al. 1998). Unlike harp and hooded seals, Weddell seals remain in similar and accessible locations throughout the reproductive and molting periods. Long-term research on the McMurdo Sound Weddell seal population (Cameron et al. 2007; Garrott et al. 2012) allows sampling of females with known reproductive histories. Additionally, they are highly philopatric (Cameron et al. 2007), thus accessing individuals for longitudinal sampling between breeding seasons makes them the ideal species for seasonal comparisons.

In Chapter 1 titled, "To each its own: Thermoregulatory strategy varies among neonatal polar phocids," I compared thermoregulatory strategy among species with differing ontogeny. I hypothesized that pups with high SA:V would need additional thermogenesis to maintain euthermia, pups with blubber may be better insulated than those with only lanugo, and pups in extreme low temperature or those that get wet would need to increase thermogenesis to maintain euthermia. To address these hypotheses, I quantified the potential contribution of insulation, NST, and muscle thermogenesis (via muscle enzyme activity) to overall thermoregulation. I then related these physiological findings to what is known about the environmental conditions (e.g., ambient temperature and substrate stability) for each species. Because there are large differences in the duration of lactation and maternal energy investment among these three species (Kovacs and Lavigne 1986; Boness and Bowen 1996; Oftedal et al. 1996; Schulz and Bowen 2004), I compared newborn pups, an equivalent developmental stage rather than a calendar age (Kovacs and Lavigne 1986). This chapter has been published in the Comparative Physiology and Biochemistry Part A.

Chapter 2 titled, "Shifts in thermoregulatory strategy during ontogeny in harp seals (*Pagophilus groenlandicus*)," examines shift in thermoregulatory strategy as pups develop and prepare for an aquatic lifestyle. Whereas this chapter focuses solely on harp seals, the pattern of thermoregulatory shifts should be similar across all species in which pups transition from lanugo coat to subdermal insulation, even if the exact timing differs. I hypothesized that as pups nurse, the primary insulation would shift from lanugo to blubber, and the need for heat production would decline, as immersion would no longer be a threat to euthermia. The morphological transition from reliance on lanugo to blubber as the primary source of insulation has been well described in harp seals (i.e., Kvadsheim and Aarseth, 2002; Oftedal et al., 1996; Worthy, 1991). However, changes in insulative ability have not previously been linked with potential heat generating mechanisms, such as NST and ST. I quantified the insulative properties of the fur and blubber during ontogeny, examined potential capacity of heat production through NST and shivering, and determined if changes in insulation were correlated with changes in the thermogenic capacity. This chapter has been published in the Journal of Thermal Biology.

In Chapter 3 titled, "Influence of biotic and abiotic factors on blubber fatty acid profiles among the pinnipeds," I compared the relative proportions of the three main FA classes (SFA, MUFA, PUFA) in blubber of phocid and otariid species using a combination of literature and

direct analysis. I hypothesized that the FA in blubber would be influenced by environmental interactions with temperature; species that experience low temperatures would have more PUFA in the blubber. Further, this increase in PUFA would particularly evident in species where the thermal gradient between core and ambient is achieved across the blubber. I then compared the FA profiles of blubber and muscle among adult harp, hooded, and Weddell seals. I hypothesized that the FA classes of muscle would be similar among species, given skeletal muscle is held in a warm thermal environment, and all three species have similar underlying muscle physiology. Finally, I determined the influence of physiological parameters (i.e., mass, body condition) on the relative proportion of FA classes of blubber or muscle in individuals from each species. This chapter has been prepared for publication in the Journal of Thermal Biology.

Chapter 4 titled, "Seasonally persistent differences in the fatty acid profiles of Weddell seal muscle and blubber," tested if the relative proportions of FA classes and individual FA differed between blubber and skeletal muscle. I hypothesized that physiological (i.e., mass, body condition, reproductive status) and behavioral (diving activity) characteristics, known to impact metabolism and energy balance, would influence the relative proportions of FA in blubber and muscle. Further, I hypothesized that there would be seasonal changes in the relative proportions of FA within each tissue, and these seasonal changes would be influenced by seasonal shifts in the physiological and behavioral characteristics listed above. To address these questions, I measured the relative proportion of FA classes and individual FA in blubber and muscle of adult female Weddell seals handled at the end of the molt period in the austral fall, and at the beginning of the following reproductive period in the austral spring, after animals have completed their overwinter foraging activities. Additionally, I compared the FA profiles of blubber and muscle between fall and spring in a subset of seals handled at both time points. This chapter has been prepared for publication in Polar Biology.

Energy allocation and behavior change throughout the life of many mammals, including phocids, because of tradeoffs among maintenance, growth, and activity (Miller and Irving 1975; Worthy 1987; Worthy and Lavigne 1987; Beauplet et al. 2003; McDonald et al. 2012). Examining how conspecifics are adapted to extreme environments, such as those found at the poles, allows better understanding of how physiological traits and modification of these traits fit into a particular suite of ecological conditions (Hochachka and Somero 2002). Overall, this is the first study to integrate the roles of varying lipid-rich tissues across the development and life of

three mammalian species, and the impact of the specific costs and benefits of these tissues at varying life stages.

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Chapter 1: To each its own: Thermoregulatory strategy varies among neonatal polar phocids [1,2]

Abstract

Cold environmental conditions and small body size often promote heat loss and may create thermoregulatory challenges for marine mammals born in polar regions. However, among polar-born phocid seal species there are variations in physical attributes and environmental conditions at birth, allowing for an interesting contrast in thermoregulatory strategy. We compared aspects of thermoregulatory strategies including morphometrics, sculp attributes (conductivity and resistance), nonshivering thermogenesis (NST via uncoupling protein 1; UCP1), and muscle thermogenesis (via enzyme activity) in neonatal harp (*Pagophilus groenlandicus*), hooded (*Cystophora cristata*), and Weddell seals (*Leptonychotes weddellii*). Harp seals are the smallest at birth (9.8 ± 0.7 kg), rely on lanugo (82.49 ± 3.70% of thermal resistance), and are capable of NST through expression of UCP1 in brown adipose tissue (BAT). In contrast, hooded seal neonates (26.8 ± 1.3 kg) have 2.06 ± 0.23 cm of blubber, accounting for 38.19 ± 6.07% of their thermal resistance. They are not capable of NST, as UCP1 is not expressed. The large Weddell seal neonates (31.5 ± 4.9 kg) rely on lanugo (89.85 ± 1.25% of thermal resistance) like harp seals, but no evidence of BAT was found. Muscle enzyme activity was highest in Weddell seal neonates, suggesting they rely primarily on shivering thermogenesis (ST). Similar total thermal resistance, combined with the marked differences in thermogenic capacity of NST and ST among species, strongly supports the idea that thermoregulatory strategy in neonate phocids is more closely tied to pups' surface area to volume ratio (SA:V) and potential for early water immersion rather than mass and ambient environmental conditions.

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[1] Pearson LE, Liwanag HEM, Hammill MO, Burns JM. 2014. To each its own: Thermoregulatory strategy varies among neonatal polar phocids. Comparative Physiology and Biochemistry Part: A 178: 59-67.

[2] Abbreviations: BAT: brown adipose tissue; COX: cytochrome c oxidase; CS: citrate synthase; FMR: field metabolic rate; HOAD: β-hydroxyacyl CoA dehydrogenase; LD: longissimus dorsi; MR: metabolic rate; MT: mitochondria; NST: nonshivering thermogenesis; PWF: post weaning fast; SA:V: surface area to volume ratio; ST: shivering thermogenesis; UCP1: uncoupling protein 1; TEM: transmission electron microscope

Keywords: blubber, harp seal, hooded seal, lanugo, nonshivering thermogenesis, thermal conductivity, thermoregulation, Weddell seal

1.1 Introduction

Thermoregulatory homeostasis can be challenging for marine mammals that live in cold and aquatic environments and is managed through a combination of behavioral and physiological mechanisms (Scholander et al. 1950; Ryg et al. 1993; Liwanag et al. 2009). Heat loss in adult marine mammals is typically reduced by lower surface area to volume ratios (SA:V) compared with terrestrial animals of similar size (Innes et al. 1990; Oftedal et al. 1991), reduced peripheral blood flow (vasoconstriction), counter-current heat exchangers (Scholander et al. 1950), and effective insulation (Scholander et al. 1950; Dunkin et al. 2005; Liwanag et al. 2012a; Liwanag et al. 2012b). A thick subcutaneous lipid depot provides insulation in adult phocid seals (Kvadsheim and Folkow 1997; Liwanag et al. 2012b). Their blubber layer protects against the cold and is effective against the increased conductivity of water experienced as a result of their aquatic lifestyle. In general, it is thought that adult phocid seals have broad thermal neutral zones as a result of this thick blubber layer (Hokkanen 1990).

Unlike adult seals, which rely on blubber, most phocid seal pups are born with little or no blubber and rely instead on a lanugo coat (Scholander et al. 1950; Ling 1974; Elsner et al. 1977; Oftedal et al. 1991; Kvadsheim and Aarseth 2002). Phocid pup fur is lighter and more insulative in air than a similar thickness of blubber; small-bodied animals can achieve greater insulation with less volume and weight using fur compared with blubber (Ryg et al. 1993). However, when phocid fur becomes wet, water replaces the warm, trapped air in the under-fur and heat can be readily conducted away from the body (Scholander et al. 1950; Davydov and Makarova 1964; Elsner et al. 1977; Kvadsheim and Aarseth 2002). Accordingly, lanugo is a good insulator for phocid species that live in dry environments or on stable substrates (i.e., elephant seals, *Mirounga angustirostris* and ribbon seals, *Histriophoca fasciata*) (Oftedal et al. 1991; Smith et al. 1991). However, it is not as effective for species with high potential for early immersion in water, as may occur in species born on unstable pack ice, or phocid species that naturally enter the water early in life (hooded seals, *Cystophora cristata* and harbor seals, *Phoca vitulina*) (Oftedal et al. 1991). In these species, pups molt the lanugo *in utero*, and are born with a subcutaneous blubber layer and a more adult-like pelage (Burns 1970; Bowen et al. 1987;

Oftedal et al. 1991). The deposition of a sufficiently thick blubber layer *in utero* can provide neonates with effective insulation even if they enter the water; therefore, lanugo is extraneous (Oftedal et al. 1991).

Neonatal phocids are precocial compared with most other terrestrial carnivores (Kovacs and Lavigne 1985; Kovacs and Lavigne 1986), but they are typically born without the heat conserving adaptations of adults (Scholander et al. 1950; Irving and Hart 1957; Blix and Steen 1979). Young phocids have a large SA:V (Blix and Steen 1979; Oftedal et al. 1991) and poorly developed vasocontrol (Lapierre et al. 2004). Yet somehow, they maintain a stable core body temperature (Scholander et al. 1950; Irving and Hart 1957; Blix and Steen 1979; Little 1995), even when ambient temperatures are well below freezing (Ortisland and Ronald 1978; Blix and Steen 1979). To maintain euthermia, young seals may employ thermoregulatory strategies not used by adults to increase their thermogenic capacity. Thermogenic capacity is determined by metabolic rate (MR), NST in brown adipose tissue (BAT) (Cannon and Nedergaard 2004), shivering thermogenesis (ST) (Davydov and Makarova 1964; Elsner et al. 1977; Blix et al. 1979), and/or futile cycling of calcium ions in muscle (de Meis et al. 2005; Arruda et al. 2007). However, these mechanisms come at a high metabolic cost (Cannon and Nedergaard 2004; de Meis et al. 2005), reducing the energy available for growth and development. Selective pressure may result in using these mechanisms sparingly (Thompson et al. 1987; Little 1995).

Although neonatal mass typically scales with maternal mass (Schulz and Bowen 2004; Wheatley et al. 2006), differences in pelage, blubber thickness, and body size are common among young phocids. For example, harp (*Pagophilus groenlandicus*) and hooded seals are born on pack ice in March when ambient air temperatures are low and storm events are common (Table 1). Yet, harp seals have a small body size (~7% of maternal mass; Table 2; Anderson and Fedak 1987), a very thin blubber layer, and a white lanugo coat that provides the primary insulation (Kvadsheim and Aarseth 2002; Pearson et al. 2014). Hooded seal pups are larger (typically 11% of maternal body mass; Table 2; Bowen et al., 1985; Anderson and Fedak, 1987), they molt their lanugo pelage *in utero*, and a blubber layer provides insulation (Bowen et al. 1985; Oftedal et al. 1991; Lydersen et al. 1997). At the opposite pole, the larger Weddell seals (*Leptonychotes weddellii*) are born on stable fast ice in October and November around Antarctica, when severe weather and storms are common (Table 1). Though large in body mass, neonatal Weddell seals are small relative to maternal mass (~6%; Table 2; Anderson and Fedak

1987; Wheatley et al. 2006), and they are born with little blubber and a wettable lanugo (Elsner et al. 1977). These three species each highlight a different characteristic of young phocids, which make them a good comparison.

Previous studies on thermoregulation in young phocids primarily focused on mechanisms preventing heat loss (Scholander et al. 1950; Blix and Steen 1979; Kvadsheim and Aarseth 2002), with little work quantifying heat-generating mechanisms. Additionally, for a given species and age class, few studies (e.g., Pearson et al., 2014) have quantified a comprehensive suite of thermoregulatory mechanisms. In this study, we used a comparative approach to quantify insulation, the capacity for NST, and muscle thermogenesis (via muscle enzyme activity) in harp, hooded, and Weddell seals. We related the primary thermoregulatory mechanism to what is known about the environmental conditions (e.g., ambient temperature and substrate stability) for each species. Further, we examined how thermoregulatory strategies differ among species with different developmental patterns. Because there are large differences in the duration of lactation and maternal energy investment among phocids (Kovacs and Lavigne 1986; Boness and Bowen 1996; Oftedal et al. 1996; Schulz and Bowen 2004), we compared neonatal pups at an equivalent developmental stage (Kovacs and Lavigne 1986). We hypothesized that harp seals may rely on thermogenesis via NST or muscle thermogenesis to maintain euthermia at birth (Davydov and Makarova 1964; Blix et al. 1979; Kvadsheim and Aarseth 2002), and hooded seal neonates may not require additional thermogenesis, as their smaller SA:V and blubber make them better insulated. We hypothesized that Weddell seals may also rely on thermogenesis via NST or muscle thermogenesis to maintain euthermia, as they are born into a harsher environment with very little blubber.

1.2 Materials and Methods

1.2.1 Sample collection

Ten harp seal (*P. groenlandicus*) neonates (within hours of birth; 6 from Canada, 4 from Greenland; 5 males, 5 females) and 8 hooded seal (*C. cristata*) neonates (within hours of birth; 5 from Canada, 3 from Greenland; 5 males, 3 females) were captured in March 2008 in the Gulf of St. Lawrence, Canada (N47°36' W62°13'), and in March 2011 in the "West Ice" off Greenland (N72°24', W14°15'). Harp and hooded seal pups were aged based on coat appearance and mass

(Stewart and Lavigne 1980; Bowen et al. 1987). Pups were sacrificed using methods approved for scientific harvest in Canada (DFO Permit: IML-2007-04) or Norway (Directorate of Fisheries under the Norwegian Ministry of Fisheries and Coastal Affairs #7764 4900). Six frozen Weddell seal (*L. weddellii*) neonate carcasses (3 males, 3 females) were opportunistically collected in October 2010 and 2011 from the McMurdo Sound region (S77°40', E166°30'). All pups were less than 2 days old as determined by the presence of the placental sac (n = 3), freshness of the umbilicus (n = 1), or exact date of birth (n = 2). Date of birth and death was recorded during regular population surveys conducted by a collaborating research group (Project B009 lead by J. Rotella and R. Garrott) and carcasses were collected within 2 days post-mortem. Pups likely froze soon after death given the ambient temperature was well below 0 °C, and were kept frozen until necropsy. All carcasses were condition Code 2 ('freshly dead'; Rowles et al., 2001). The University of Alaska Anchorage Institutional Animal Care and Use Committee approved all animal-handling protocols (Canada: #Burns2005; Greenland: #149278-1). Samples were collected and imported under NMFS Permits #782-1694-02 (Canada), #15510 (Greenland), and #87-1851-04 (Antarctica). Research activities while at McMurdo Station, Antarctica were approved under the Antarctic Conservation Act permits.

1.2.2 Morphometrics

Harp and hooded seals in both Canada and Greenland were weighed (± 0.5 kg) using a spring scale. Weddell seal mass was estimated using species-specific growth equations based on length and girth (Bryden et al. 1984). For all species, blubber depth was averaged from post-mortem measurements at 6 locations along the body (i.e., neck, axillary, sternum, mid-seal, umbilicus, and pelvis) using a ruler (± 0.1 cm). Dorsal standard length (± 0.1 cm), curvilinear length (± 0.1 cm), and girth (± 0.1 cm) (Mammalogists 1967) at each point along the body were measured with a flexible tape measure. Total body volume, percent blubber by volume, and surface area were calculated following the truncated cones method outlined in Gales and Burton (1987).

1.2.3 Insulation

We assessed the quality of insulation provided by the blubber and fur for each neonate by measuring the thermal conductivity (k; W m^{-1} $°C^{-1}$) and calculating the thermal resistance (R; m^2 °C W^{-1}) of each tissue (Kvadsheim et al. 1994; Liwanag et al. 2012a; Liwanag et al. 2012b).

Sculp samples (full blubber thickness including skin and fur) from harp and hooded seals were collected from the dorsum, just caudal to the shoulders, within 30 min post-mortem and stored at -20 °C. Weddell seals sculp samples were collected from the dorsum, just caudal to the shoulders, during necropsy and stored at -80 °C. Thermal conductivity of the sculp, blubber, and fur including skin was concurrently measured on square (~10 cm × 10 cm) sections using the 'standard materials method' described by Dunkin et al. (2005) and Liwanag et al. (2012a, 2012b). Fur was cleaned with water and dried using a hair dryer on the cool setting to restore the air layer (Liwanag et al., 2012a). Mean values of triplicate measures of the thickness measurements (± 0.01 mm) of the blubber, skin, and dry fur were taken using digital calipers (ABSO-LUTE Digimatic Caliper Series 500, Mitutoyo, Aurora IL, USA).

Conductivity samples were placed in a heat flux chamber that consisted of a well-insulated lower compartment heated to 37 °C with a circulating water bath, and an ice chilled upper compartment (~ -1 °C; Figure 1; Liwanag et al. 2012a, 2012b). The standard material, an elastomer of known conductivity (Plastisol vinyl; Carolina Biological Supply, Burlington, NC, USA; $k = 0.109$ W m^{-1} °C^{-1}), was placed on the heat source below the sample, which was exposed to the chilled air. The standard and sample were surrounded by styrofoam insulation to ensure unidirectional heat flow. Temperatures were measured simultaneously using 12 copper-constantine (Type T) thermocouples (Physitemp Instruments Inc. Clifton, NJ, USA) placed in triplicate between 1) the surface of the heat source and the standard material, 2) the standard and the sample, 3) the skin and the blubber, and 4) on top of the fur (Figure 1). All thermocouples were wired to a Fluke Hydra data logger (model 2625A; Fluke Inc., Everett, WA, USA) that recorded temperature every 10 s. The final 30 min of each trial (minimum 2 h) were used for data analysis.

Thermal conductivity was calculated across the sculp, blubber, and fur including skin using the Fourier equation (Kreith 1958):

$$H = k \times A \times \Delta T \times L^{-1} \quad \text{(Eq. 1.1)}$$

where H is the heat transfer (J s^{-1}), k is the conductivity (W m^{-1} °C^{-1}), A is the area (m^2) through which the heat is moving, ΔT is the temperature differential (°C) across the material, and L is the thickness of the material (m). Assuming heat transfer (H) is equal across the standard material and sample, the Fourier equations for both materials can be set equal and solved for the thermal conductivity of the sample. Because thermocouples were placed at the interface of each layer, we

could calculate *k* for the sculp, blubber, and fur including skin using the data from a single trial. To account for changes in insulation due to differences in the thickness of blubber and fur among species, we calculated the thermal resistance (*R*; m^2 °C W^{-1}) of the sculp, blubber, and fur including skin separately, using the equation:

(Eq. 1.2) $R = L \times k^{-1}$

1.2.4 Metabolic heat production

We wanted to assess the capacity for metabolic heat production by the *longissimus dorsi* (LD), a large and major swimming muscle, through shivering thermogenesis or futile cycling. Specifically, we measured the activity of three metabolic enzymes that play important roles in providing reducing substrates for heat production: citrate synthase (CS) as an estimate of tricarboxylic acid cycle activity, β-hydroxyacyl CoA dehydrogenase (HOAD) as a measure of lipid β-oxidation, and cytochrome c oxidase (COX) as a measure of electron transport chain activity. Samples of LD (100 mg) from harp and hooded seals were collected within 30 min post-mortem, immediately frozen in liquid nitrogen, and stored at -80 °C until assayed. Weddell seal samples were not available due to the unknown time and cause of death. Enzyme activities for neonatal Weddell seals were obtained from published values (Kanatous et al., 2008). We measured all enzyme activities under substrate saturating conditions following previously published protocols (Kanatous et al. 2008; Prewitt et al. 2010).

LD samples were homogenized at 0 °C in buffer (1:20 wt:vol) containing 1X phosphate buffered saline, 1% Tween 20, 20% glycerol, and a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) and were centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was used for the enzyme assays, which were run in a Molecular Devices SpectraMax 340 microplate reader (Sunnyvale, CA, USA) held at approximate seal body core temperature (37 °C). Assay conditions were as follows: 1) CS (EC 4.1.3.7): 50 mmol L^{-1} imidazole, 0.25 mmol L^{-1} 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 0.4 mmol L^{-1} acetyl CoA and 0.5 mmol L^{-1} oxaloacetate, pH 7.5 at 37 °C; ΔA_{412}, $\in_{412}$=13.6. 2) COX: 0.1 mmol L^{-1} DTT, 0.22 mmol L^{-1} ferrocytochrome-c, 10 mmol L^{-1} Tris-HCl pH 7.0 with 120 mmol L^{-1} KCl; ΔA_{550} $\in_{550}$=21.84. 3) HOAD (EC 1.1.1.35): 50 mmol L^{-1} imidazole, 1 mmol L^{-1} EDTA, 0.1 mmol L^{-1} acetoacetyl CoA, and 0.15 mmol L^{-1} NADH, pH 7.0 at 37 °C; ΔA_{340}, $\in_{340}$=6.22. Specific enzyme activities (IU g^{-1} wet tissue mass) were calculated from the change in absorbance during the linear slope of

the assay. Samples were run in triplicate; values were only accepted if replicate coefficients of variation (CV) were < 10%. Activity values for the entire assay plate were rejected and rerun if CVs were > 10% or if values for a control muscle with known enzyme activity fell outside a previously determined normal range published in Prewitt et al. (2010). Enzyme activity was scaled to previously published field metabolic rates (FMR) for neonates of each species (harp seal: 644.8 kJ kg^{-1} d^{-1}, Lydersen and Kovacs, 1996; hooded seal: 714 kJ kg^{-1} d^{-1}, Lydersen et al., 1997; Weddell seal: 244 kJ kg^{-1} d^{-1}, Elsner et al., 1977) prior to interspecific comparisons to control for differences in body size and metabolic rate.

1.2.5 Nonshivering thermogenesis

We examined pups of all species for evidence of BAT tissue that matched previously published descriptions of BAT from the venous plexus of the neck (Grav et al. 1974; Blix et al. 1975). Tissue samples were stored in Whirlpaks® at -20 °C for up to 2 weeks before being stored at -80 °C until analysis. Samples of muscle (LD) and blubber were also collected for use as negative controls. No BAT tissue was found in Weddell seal neonates; therefore, subsequent analyses on BAT and UCP1 were limited to harp and hooded seals.

BAT characterization and UCP1 detection

Western blot analyses were performed on 12% SDS-PAGE gels with tissue homogenates of the BAT-like tissue samples to determine if uncoupling protein 1 (UCP1) was expressed. Samples were homogenized in the same buffer used for enzyme assays and centrifuged at 10,000 G for 10 min at 4 °C. Total protein content (mg / ml) of the homogenate was determined using Pierce Coomassie Blue 'The Better Bradford' Total Protein Assay (Pierce Chemicals, Rockford, IL, USA). Thirty micrograms of protein per sample was mixed with loading dye, loaded onto 12% SDS-PAGE gels, run at 100 V for 1 h, and transferred onto a nitrocellulose membrane. Membranes were then stained with Ponceau S Solution (0.2% v/v in 5% acetic acid; Alfa Aesar, Ward Hill, MA, USA) to ensure proper protein transfer. Western blot development was done on a SNAP i.d. (EMD Millipore, Billerica, MA, USA). The primary antibody (rabbit anti-UCP1, IgG, 1:3000; #ab10983, Abcam, Cambridge, MA, USA) was detected using an Alexa Fluor 680 goat anti-rabbit (IgG, 1:5000) secondary antibody (Invitrogen, Carlsbad, CA, USA). Additionally, β-Actin (rabbit anti-β-Actin, IgG, 1:3000, #ab8227, Abcam, Cambridge, MA, USA) was detected on the same membranes, to ensure equal protein loading across samples and

gels. Protein bands were visualized using a LI-COR Odyssey imaging system (LI-COR, Lincoln, NE, USA), and band intensity was quantified using a digital analysis program (Image J, Schneider et al., 2012). Samples were run in triplicate and expression of UCP1 and β-Actin was calculated for each individual. UCP1 expression relative to β-Actin expression was calculated for each individual and averaged for each of the two species. Arctic ground squirrel (*Urocitellus parryii*) BAT was used as a positive control for antibody reactivity to UCP1 (Barger et al. 2006). We tested for cross-reactivity of the antibody with other UCPs by performing Western blot analyses using the UCP1 antibody on muscle and blubber of all three phocid species. Peptide competition assays were also performed on the BAT-like tissue to ensure binding was not due to antibody cross-reactivity. In this assay, UCP1 antibody (1:1500) was pre-incubated with UCP1 peptide (1:60; #ab24282, Abcam, Cambridge, MA, USA) for 60 min at 37 °C. Incubation and development were performed as above using the primary antibody-peptide mix in place of the primary antibody. Peptide competition resulted in complete inhibition of antibody activity, indicating that the protein bound by the UCP1 antibody in the Western blot was UCP1 and not antibody cross-reactivity.

Histology

To measure the volume density of lipid (%) in BAT and blubber, frozen samples (0.5 cm × 0.5 cm) were thawed, fixed in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 12 h at 4 °C, dehydrated with gradually increasing concentrations of ethanol (75%, 95% and 100%), cleared with Clarification NeoClear (EMD Chemicals Inc, Gibbstown, NJ, USA), and embedded in paraffin wax. Serial sections, 7 μm thick, were cut with a microtome, placed onto glass slides, dried for 12 h at 25 °C, and stained with Hematoxylin and Eosin. Slides were imaged using a Leica DM6000B microscope and Leica DFC350FX camera system (Leica Biosystems, Buffalo Grove, IL, USA). Lipid droplet volume density was determined by standard point counting procedures outlined in Weibel (1979) and modified for digital photography (Watson et al. 2007) using Adobe Photoshop CS5 (Adobe Systems Inc., San Jose, California, USA) with the 'grid' feature enabled to generate a grid of appropriate point density for the lipid droplet size of the BAT or blubber. A minimum of six images containing 400–600 test points each were counted for each tissue per individual animal.

Transmission electron microscopy

The mitochondrial volume density in BAT was measured using transmission electron microscopy (TEM) to assess tissue ultrastructure in harp and hooded seals. In the field, 15 mg subsamples of BAT-like tissue were immediately fixed in a 2% glutaraldehyde-2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and stored at 4 °C. Subsequently, at the Electron Microscopy Lab at the University of Maine (Orono, ME), samples were cut into 1 mm blocks, rinsed 3x and held overnight in 0.1 M cacodylate buffer (pH 7.4). Samples were then post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h while on ice, rinsed in DIH_2O, and dehydrated with increasing concentrations of acetone (50%, 70%, 95%, and 100%). Samples were infiltrated with 50/50 mix of 100% acetone and epon-araldite resin overnight, moved to a fresh resin under vacuum (2 x 30 min) the following day, embedded in fresh epon-araldite resin, and cured for 48 h at 65 °C. Thin sections (2 μm) were cut on a Leica UC6 Ultramicrotome (Leica Biosystems, Buffalo Grove, IL, USA) and placed onto G200 copper grids (Electron Microscopy Sciences, Hatfield, PA, USA). Grids were counter-stained with 1% uranyl acetate in water for 20 min followed by 0.5% lead citrate in water for 4 min. Sections were photographed on a JEOL 1200 Transmission Electron Microscope (JEOL, Peabody, MA, USA) in the Advanced Instrument Laboratory at the University of Alaska Fairbanks. Carbon grating replica calibration was performed on the microscope to confirm magnification was within 5% of nominal magnification. Digital images were captured at 3000x. Twenty images were taken per sample for analysis. The volume fraction of mitochondria was estimated using standard point counting procedures (Weibel, 1979) modified for digital photography (Watson et al., 2007). Adobe Photoshop CS5 (Adobe Systems Inc., San Jose, California, USA) with the 'grid' feature enabled was used to generate a grid of appropriate point density for each tissue based on the relative size of the mitochondria (Weibel, 1979). All points falling on mitochondria were counted. The relative standard error (RSE) of the volume density of each sample was calculated by pooling counts from all images for a sample and applying the RSE equation for binomial counts (Mathieu et al. 1981). RSE was 18.77 ± 4.37% for all samples.

Enzyme activity

We evaluated the metabolic flux through mitochondria in BAT by measuring the aerobic enzyme activities (μmol ml^{-1} g^{-1} wet tissue mass) of CS, HOAD, and COX using the same

protocols as described above for LD muscle. Enzyme activities in BAT and muscle were compared to assess the metabolic flux and potential for heat generation of each tissue.

1.2.6 Statistical analysis

Data were tested for normality and equal variance. No data points were identified as outliers (mean ± 2 SD), and all data points were retained. One-way ANOVAs and Bonferroni post-hoc tests were used to determine differences among species, and differences were considered significant at the 95% level ($p < 0.05$). Means are reported ± SEM. All analyses were completed in SPSS Software (ver. 21, IBM, Armonk, NY, USA).

1.3 Results

1.3.1 Morphometrics

The mean mass values of pups sampled in this study (Table 2) fall within previously published birth-mass range (harp: 10.8 ± 0.7 kg, Stewart and Lavigne, 1980; hooded: 24.1 ± 0.9 kg, Oftedal et al., 1989; Weddell: 30.4 ± 0.5 kg, Elsner et al., 1977; Hill, 1987) as do measurements of standard length (harp: 84.6 ± 2.7 cm, Stewart and Lavigne, 1980; hooded: 103.0 ± 6.4 cm, Oftedal et al., 1989; Weddell: 123.0 ± 1.3 cm, Burns, 1997). Because values fell within established ranges for neonates for each species, all pups in this study were considered neonates.

There were significant differences among species in the mass ($F_{2,20} = 31.93$, $p < 0.001$), standard length ($F_{2,20} = 30.93$, $p < 0.001$), total volume ($F_{2,11} = 15.57$, $p = 0.001$), surface area ($F_{2,11} = 21.73$, $p < 0.001$), and SA:V ($F_{2,11} = 8.85$, $p = 0.005$) of neonates. Post-hoc tests revealed that Weddell seal and hooded seal neonates were ~3x heavier and 1.2–1.3x longer than harp seals (Table 2). Although mass and surface area did not significantly differ between Weddell and hooded seals, Weddell seals were 1.2x longer (Table 2), and thus had significantly higher SA:V ($p = 0.01$). Blubber volume ($F_{2,11} = 66.39$, $p < 0.001$) and % blubber by volume ($F_{2,11} = 81.85$, $p < 0.001$) also significantly differed among the three species (Table 2). Weddell seals had the lowest % blubber by volume and % lipid in the blubber of all the species (Table 2). Hooded seals were born with 3x the blubber volume of the other two species (Table 2).

1.3.2 Insulation

The thermal conductivity of the intact sculp ($F_{2,12} = 1.18$, $p = 0.341$), the blubber layer alone ($F_{2,12} = 0.20$, $p = 0.824$), and the fur including skin portion did not differ significantly among species (Table 3). Thermal resistance, which accounts for differences in thickness of each tissue, did not differ significantly for the full sculp or the fur including skin (Figure 2). However, hooded seal blubber had significantly greater resistance ($p < 0.001$) than the blubber of either harp or Weddell seal neonates. The proportion of total thermal resistance attributed to the blubber versus the fur including skin was significantly higher ($F_{2,11} = 13.39$, $p = 0.001$) in hooded seals (39.2 ± 6.1%) than harp (17.5 ± 3.7%; $p = 0.007$) and Weddell seals (10.2 ± 1.3%; $p = 0.001$).

1.3.3 Metabolic heat production

Metabolically scaled enzyme activity (Table 4) showed that Weddell seals had substantially greater potential to produce heat via shivering, as they possessed significantly higher CS/FMR ($F_{2,12} = 10.90$, $p = 0.002$) and HOAD/FMR ($F_{2,12} = 3710.96$, $p < 0.001$), but not COX/FMR ($F_{2,12} = 3.58$, $p = 0.060$) among species (Table 4).

1.3.4 Nonshivering thermogenesis

We collected tissue that visually resembled BAT from the venus plexus region of the neck in harp and hooded seals. A macroscopically comparable tissue was not observed anywhere in the body of Weddell seals, though several tissue samples were collected from the same region as harp and hooded seals. UCP1 was only expressed in harp seal BAT tissue (Figure 3a). No UCP1 was detected in muscle, blubber, or other tissue, and no cross-reactivity with other UCPs occurred in any tissue of any of the three species studied. TEM and histological analysis of the harp seal BAT revealed that it contained 14.03 ± 2.02% mitochondria and 79.83 ± 3.37% lipid in multilocular droplets distributed through the cells (Table 4; Figure 4a). Activity of CS, COX, and HOAD in BAT was significantly higher than in the LD ($F_{1,15} = 11.97$, $p = 0.004$; $F_{1,15} = 6.44$, $p = 0.023$; and $F_{1,15} = 12.97$, $p = 0.003$, respectively; Table 4) indicating a greater potential for lipid-fueled heat generation in BAT than muscle.

In hooded seals, the tissue that resembled BAT macroscopically did not express UCP1 (Figure 3b), contained ten-fold fewer mitochondria (1.40 ± 0.01%; $F_{1,6} = 14.22$, $p = 0.009$; Table 4; Figure 4b), and did not have elevated enzyme activities compared with LD, and as such is

essentially a white adipose tissue (WAT) deposit. Indeed, the WAT had similar (COX; $F_{1,13} = 0.23$, $p = 0.641$) or significantly lower (CS $F_{1,13} = 16.27$, $p = 0.001$; and HOAD $F_{1,13} = 29.24$, $p < 0.001$) enzyme activity than the LD (Table 4), indicating less potential for heat generation in WAT than in muscle. In addition, while the lipid content of the BAT in harp seals and WAT in hooded seals was similar, (Table 4; $F_{1,6} = 0.42$, $p = 0.539$), the cells in the WAT of hooded seal neonates were typically unilocular (Figure 4b), and enzyme activity was lower compared with the BAT in harp seals (COX: $F_{1,13} = 5.64$, $p = 0.034$; CS: $F_{1,13} = 23.72$, $p < 0.001$; and HOAD: $F_{1,13} = 17.24$, $p = 0.001$; Table 4).

1.4 Discussion

This study compared the insulation and mechanisms contributing to thermogenic capacity in three polar phocid species: harp, hooded, and Weddell seals. There was no difference in the conductivity of the blubber layer, despite differences in the amount of lipid (Table 3 and Table 2). Differences in insulation did arise in the extent to which the blubber and fur contributed to the overall insulation (resistance). Both Weddell seals and harp seals relied on lanugo, and the thin subcutaneous blubber layer provided little insulation, whereas in hooded seals, the subcutaneous blubber greatly contributed to their overall thermal resistance. However, surprisingly, despite differences in the contribution of blubber versus fur (Figure 2), ambient conditions (Table 1), and morphometrics (Table 2), neonatal harp, hooded, and Weddell seals were similarly insulated at birth (i.e., similar total thermal resistance; Figure 2). As amounts of insulation did not differ among species, the drivers of thermoregulatory capacity were pups' SA:V, potential for immersion, and the presence or absence of lanugo.

While all three species had similar insulation, harp seals have a high SA:V (Table 2) and they likely experience a high rate of heat loss, particularly if their lanugo is wet. Indeed, neonatal harp seals shiver right after birth when their coat is soaked with amniotic fluid (Blix et al., 1979); however, muscle glycogen reserves become depleted within hours and shivering is not observed further (Blix et al., 1979). Previous studies found evidence of BAT-like tissue in the venous plexus of the neck of harp seals (Grav et al. 1974; Grav and Blix 1976; Blix et al. 1979), and our work (including Pearson et al., 2014) shows harp seals express UCP1 in BAT. This makes harp seals the second small polar phocid known to be capable of NST, after the ringed seal (*Pusa hispida*) (Taugbøl 1982). The low aerobic capacity in the LD (this study) and 5 other skeletal

muscles (Burns et al., accepted PBZ) and the expression of embryonic muscle fibers (J. Burns unpublished data) suggest that the skeletal muscle of harp seals is not sufficiently developed for the constant endurance activity of ST (Asakura 2004; Cannon and Nedergaard 2011). Additionally, harp seal neonates are likely aided by the liberation of heat as a result of their high FMR and relatively fast growth rate during the nursing period (Worthy 1987; Lydersen and Kovacs 1996). Alternatively, the acute cold exposure may result in an increase in the thermogenic capacity of BAT, and NST will compensate for the cold demand, allowing shivering to cease as occurs in cold exposed mice (Cannon and Nedergaard 2011). However, we cannot assess the total capacity for heat generation via NST because norepinephrine challenges (Cannon and Nedergaard 2004) have not been performed, and we cannot account for the metabolic effects of norepinephrine on other tissues (Cannon and Nedergaard 2011). In terrestrial species (mouse, rat), BAT can increase metabolic rates about 4x (Cannon and Nedergaard 2004). Davydov and Makarova (1964) showed the MR of neonatal harp seals increases 2.4x after immersion in 0 °C water; this increase in MR may represent, in part, the activation of BAT. Therefore, while harp seal pups may use NST, it is costly. Indeed, storms, rain, early ice break-up, and water-immersion, all of which may increase thermogenesis, are all known to dramatically reduce pup survival (Friedlaender et al. 2010; Bajzak et al. 2011; Johnston et al. 2012). Thus, neonatal harp seal survival may be closely linked with the probability of getting wet at a very young age before a subcutaneous blubber layer is established (Pearson et al. 2014), and of the three species studied, harp seals may be the most vulnerable to climate changes.

While intermediate in absolute size, hooded seal neonates had the lowest SA:V, and were the only species born with thick subcutaneous blubber (Table 2), and no lanugo coat. Their blubber does not make them better insulated in air, as they had equivalent total thermal resistance to harp and Weddell seals. However, because blubber accounts for a large proportion of their overall resistance, immersion may not present a severe thermal challenge to hooded seals, despite being born on unstable pack ice (Liwanag et al. 2012b). In addition, hooded seal pups grow very quickly during their short nursing period, gaining 20–25 kg over 3.6 days (Table 2; Lydersen et al., 1997; Mellish et al., 1999), and they have the highest mass-specific FMR (Lydersen et al. 1997) of all three neonate species in this study. Quick growth and high mass-specific FMR would naturally result in the liberation of metabolic heat (Bowen et al., 1985;

Lydersen et al., 1997), contributing to the thermal balance of the pups. In combination, the absence of functional BAT and the relatively low muscle enzyme activity suggest the low SA:V and thick blubber layer of neonatal hooded seals are sufficient to meet most thermoregulatory challenges. This is likely essential, as any need for additional thermogenesis, and subsequent increased MR during the extremely brief nursing period, would be detrimental to the energy savings necessary to fuel and maintain the post-weaning fast. While an evolutionary comparison of three species is unwarranted, it is worth noting that hooded seals, the most basal of the three species on the phocid phylogeny (Higdon et al. 2007), have what is considered to be an advanced thermoregulatory adaptation as they are born with blubber, which is considered to be an advanced marine mammalian trait (for discussion see Liwanag et al. 2012b). Additionally, the non-functional BAT appears to be an evolutionary remnant left over by selection for a thermal strategy associated with the extremely short weaning time and potential for early immersion.

Though neonatal Weddell seals are the largest by mass of the 3 species studied, they have a high SA:V and are born with lanugo. In the Antarctic there is little precipitation (Table 1), and pups are born on stable fast ice, reducing the risk of immersion and soaking the lanugo. As in earlier studies (Hammond et al. 1971; Elsner et al. 1977), we found no evidence of BAT or UCP1 expression in Weddell seal neonates. Because UCP1 is the only member of the uncoupling protein family known to have thermogenic activity (Barger et al. 2006; Golozoubova et al. 2006), neonatal Weddell seals are likely incapable of NST, despite previous speculations that NST might be important in this species (Noren et al. 2008; Trumble et al. 2010). Unlike harp seals, Weddell seal pups shiver throughout the nursing period (Ray and Smith 1968; Elsner et al. 1977). While the enzyme values used in proxy from Kanatous et al. (2008) are from older Weddell seal pups, recent work by Burns et al. (in press) shows there is no significant difference in the absolute enzyme activity in LD over the nursing period of harp or hooded seals. Further, Geiseler (2011) showed enzyme activity did not increase substantially in LD of hooded seals until pups were ~3 mo old, well after animals began foraging and diving independently. We hypothesize a similar developmental trend in muscle enzyme activity is likely in Weddell seal pups. Additionally, even if the absolute enzyme values used here represent a 20% increase from neonate levels (similar to the trend in harp and hooded seal pups over the nursing period), the metabolically scaled enzyme activity neonate Weddell seals would still be significantly higher than harp or hooded seals. Indeed, Burns et al. (accepted PBZ) found the greatest changes in

muscle metabolic activity occurred because of the lower FMR of older pups rather than a change in the enzyme activity. High metabolically scaled enzyme activity, combined with previously reported greater volume density of mitochondria and expression of calcium handling proteins in the LD compared with adults (Kanatous et al. 2008), all suggest increased muscle metabolism and futile-cycling based thermogenesis (de Meis et al. 2005; Arruda et al. 2007; Kanatous et al. 2008). This also suggests Weddell seal neonates, unlike harp seals, are born with muscles equipped for shivering, although this has yet to be studied directly. Weddell seal pup LD also has a greater proportion of polyunsaturated fatty acids (PUFAs) (Trumble et al. 2010), which is associated with higher protein activity and metabolic rate (Hulbert and Else 2005). UCP1-ablated mice acclimated to temperatures just below their thermal neutral zone (TNZ) have enhanced capacity for ST, which compensates for the lack of NST when animals are held at temperatures well below their TNZ (Cannon and Nedergaard 2011). Such may be the case if Weddell seals are born in temperatures at the lower limit of the TNZ, and subsequently exposed to inclement weather. Although the FMR reported for Weddell seals is low compared with the other two species in this study, it is still ~ 2 x the MR predicted by Kleiber's equation for a terrestrial mammal of similar size (Elsner et al. 1977). Heavy reliance on metabolic heat generation when ambient temperatures are below their lower critical temperature likely has a high energetic cost, which may contribute to their long nursing period and relatively slow growth and weight gain (Table 2; ~2 kg d^{-1}, Hill, 1987; Tedman and Bryden, 1979; Wheatley et al., 2006).

The similar total thermal resistance among harp, hooded, and Weddell seals, combined with the marked differences in thermogenic capacity of NST and ST, strongly supports the idea that thermoregulatory strategy in neonates is more closely tied to the pups' SA:V and potential for early water immersion rather than mass and ambient conditions. Indeed, species that begin swimming during the nursing period, such as bearded seals *Erignathus barbatus* (Hammill et al. 1994; Lydersen et al. 1994; Gjertz et al. 2000) and harbor seals (Kovacs and Lavigne 1986; Oftedal et al. 1991), are born with a thick subcutaneous blubber layer (Oftedal et al. 1991; Lydersen et al. 1994; Kovacs et al. 1996), and as a result are larger as a percent of maternal mass (16% and 13% respectively; Kovacs and Lavigne, 1986). This decreases their SA:V, and in combination, likely protects against the thermal challenge in water (Lydersen et al. 1994; Kovacs et al. 1996). Like hooded seals in this study, the combination of lower SA:V and blubber may preclude thermogenesis. However, there are constraints on the amount of blubber that a neonate

can have at birth. For example, the conductivity of the blubber is approximately double that of the lanugo in harp seals; to achieve equivalent insulation with blubber alone, harp seal neonates would need to be born with 4 cm of blubber, 5x thicker than the blubber with which they are born (Table 2). Thus, if harp seals were born with enough blubber to provide the equivalent insulation of their lanugo coat, neonatal body composition would be over 75% blubber; this larger size and body mass would probably exceed that which could be safely carried and birthed. More conservatively, harp seals would need 2 cm of blubber for it to account for 40% of the resistance, which is equivalent to the insulation provided by blubber in neonatal hooded seals. Similarly, Weddell seal pups would need to be born with 3.5 cm of blubber (7x thicker than the blubber with which they are born; Table 2) to have insulation equivalent to that provided by the fur, or 1.6 cm blubber for 40% of resistance. For species born with lanugo and high potential for immersion, such as harp, spotted (*Phoca largha*), and ringed seals (*P. hispida*), NST or ST is likely essential for maintaining euthermia and drying the coat (Blix and Steen 1979; Smith et al. 1991). However, because this comes at a high metabolic cost, heavy reliance on such thermogenic mechanisms as NST or ST will cause slow growth and potentially lower juvenile survival.

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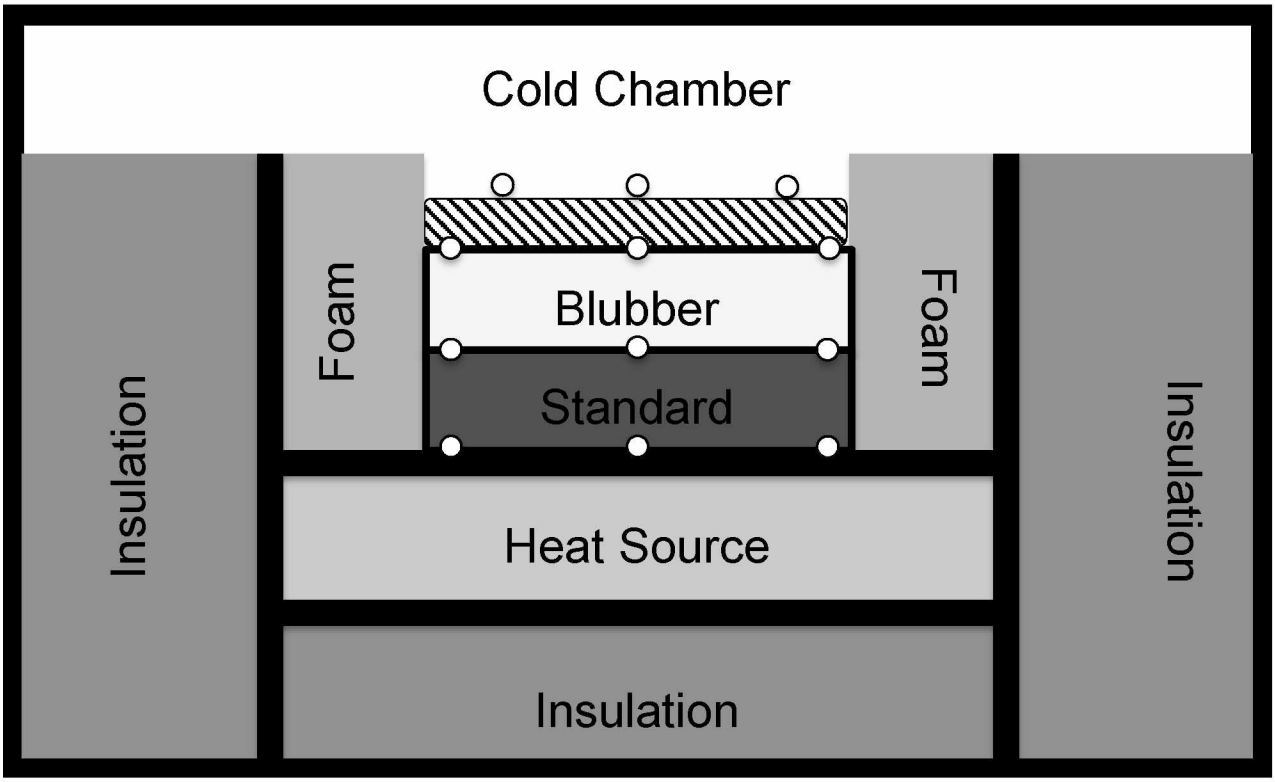

Figure 1.1: Conductivity chamber set-up. The relative positions of the sculp sample, standard material, and thermocouples (open circles) are shown. Diagonal lines represent the fur layer of the sample. Adapted from Mostman Liwanag (2008).

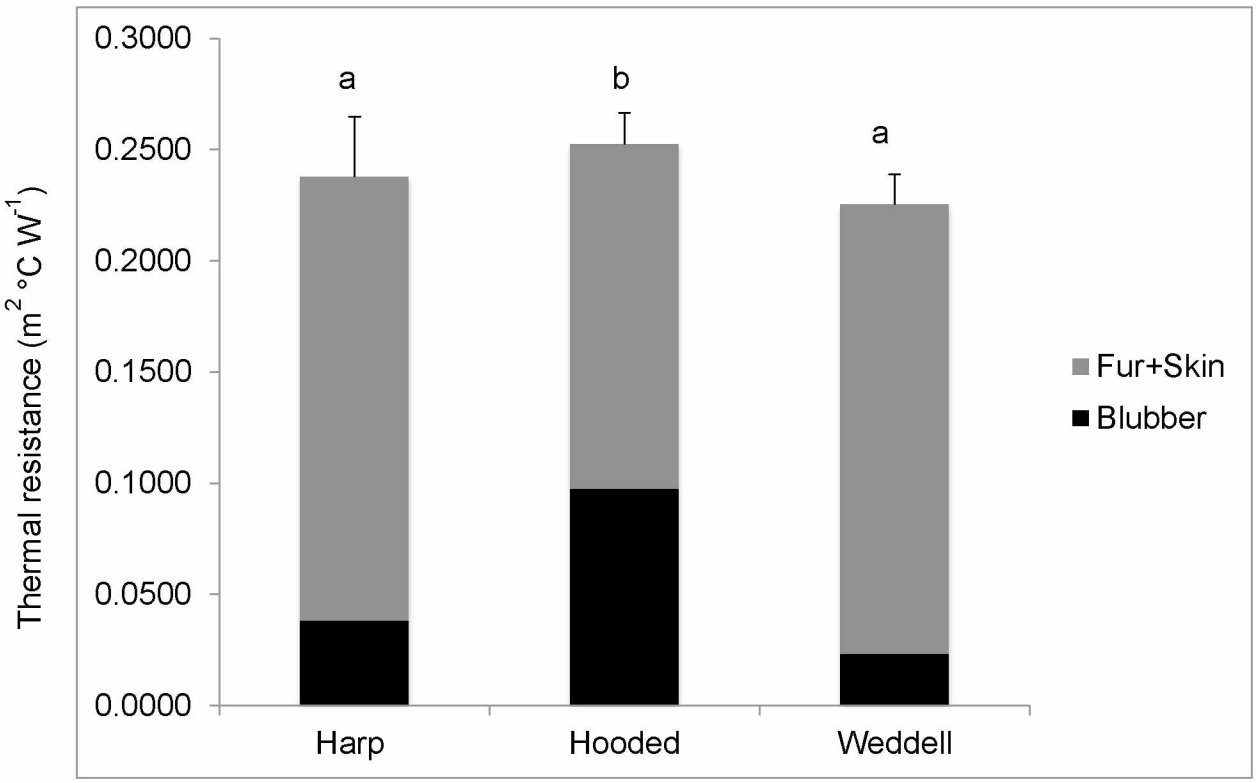

Figure 1.2: Total thermal resistance (m °C W^{-1}) of harp, hooded, and Weddell seal neonates (mean ± SEM). Total thermal resistance is the sum of the thermal resistance of the blubber (black bar) and pelt (fur + skin, gray bar). There was a significant difference in the contribution of blubber to the overall resistance ($p < 0.05$), although the resistance of fur + skin was not significantly different ($p > 0.05$) among species. Letters indicate differences among species where there was a significant change in the resistance of blubber.

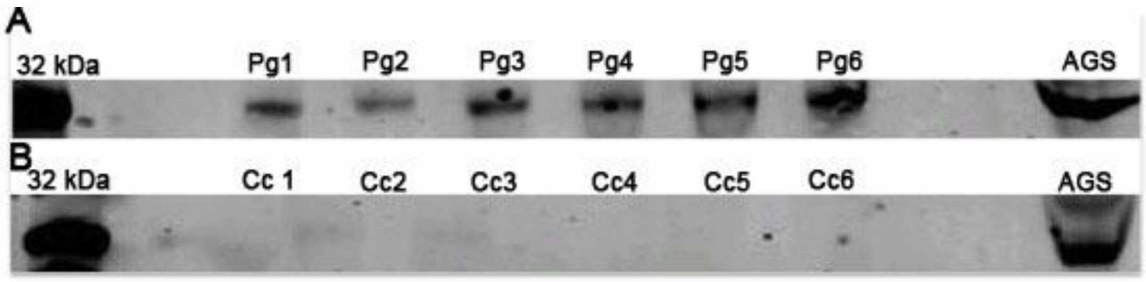

Figure 1.3: Representative western blots for uncoupling protein 1 expression. Brown adipose tissue of harp (A) and hooded (B) seal neonates. No brown adipose tissue was found in Weddell seal neonates. Each band represents an individual seal. AGS represents the positive control, Arctic ground squirrel brown adipose tissue.

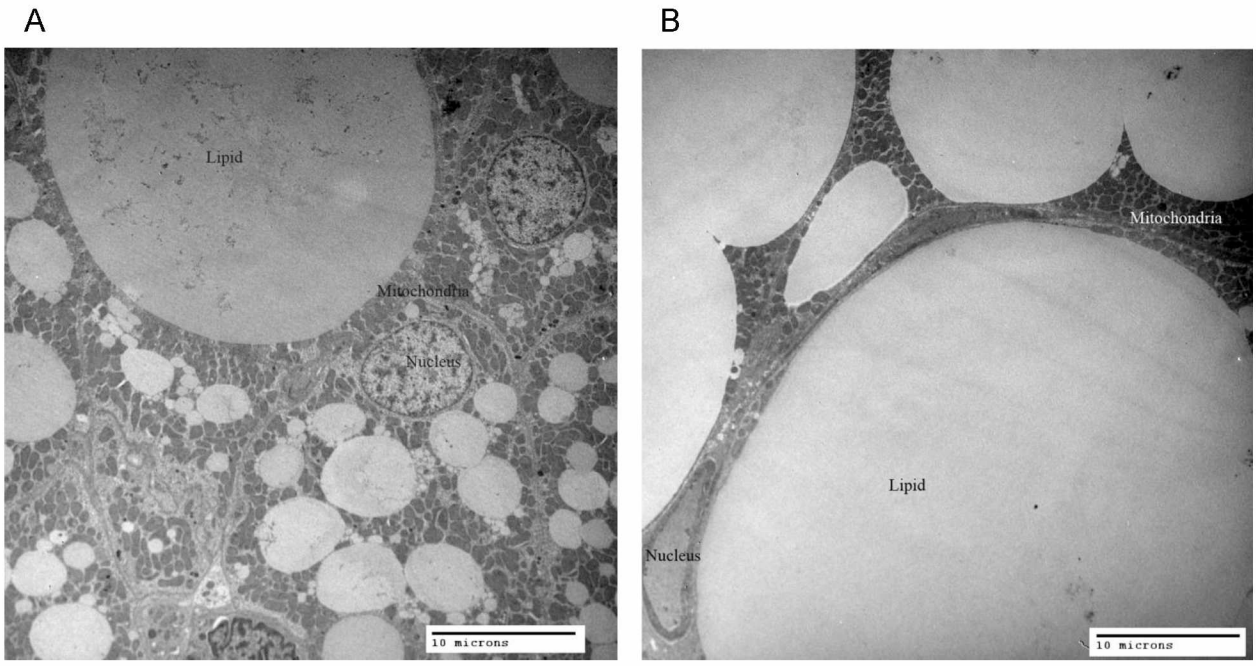

Figure 1.4: Transmission electron microscopy of the ultrastructure of brown adipose tissue. From harp seal neonates (A), and the brown adipose-like tissue from hooded seal neonates (B). Note that the harp seal brown adipose tissue consists of multilocular cells with many mitochondria present, whereas the brown adipose-like tissue in hooded seals contains fewer mitochondria, and consists of large unilocular and more lipid dense cells. No brown adipose tissue was found in Weddell seals.

Table 1.1: Environmental variables (mean ± SD) from 2003 to 2013. Data for the three sampling locations (Gulf of St. Lawrence, Canada, "West Ice", Greenland, McMurdo Sound, Antarctica) during the peak pupping dates by location for harp, hooded, and Weddell seals.

	Gulf of St Lawrence[1]	**"West Ice"**[2]	**McMurdo Sound**[3]
Species	Harp/hooded	Harp/hooded	Weddell
Birth substrate	Pack ice	Pack ice	Fast ice
Ambient temperature (°C)	-5.13 ± 4.28	-2.80 ± 3.84	-15.28 ± 3.11
Minimum temperature (°C)	-7.90 ± 4.82	-4.71 ± 3.93	-18.99 ± 3.41
Maximum temperature (°C)	-2.21 ± 4.44	-1.02 ± 3.49	-12.12 ± 3.20
Daily precipitation (mm)	3.36 ± 6.47	1.57 ± 2.54	0.13 ± 0.49
Total precipitation (mm)	30.85 ± 15.61	15.62 ± 11.75	2.07 ± 1.51

[1] Data from Environment Canada: Climate (http://climate.weather.gc.ca/)
[2] Data from Norwegian Meteorological Institute (http://met.no/English/)
[3] Data from Antarctic Meteorological Research Center, University of Wisconsin–Madison

Table 1.2: Condition indices of neonatal harp, hooded, and Weddell seals (mean ± SEM). Published values on maternal mass, length of nursing period, and pup growth rate are also included.

	Harp seal	Hooded seal	Weddell seal
Mass [kg]	9.8 ± 0.7[a]	26.8 ± 1.3[b]	31.5 ± 4.9[b]
Standard length [cm]	82.8 ± 2.8[a]	100.7 ± 2.2[b]	122.2 ± 6.2[c]
Surface area [m^2]	325.2 ± 13.9[a]	616.4 ± 39.1[b]	572.6 ± 32.3[b]
Total volume [L]	11.05 ± 0.82[a]	34.41 ± 3.17[b]	21.07 ± 2.94[c]
SA:V	29.62 ± 0.99[b]	18.05 ± 0.51[a]	28.80 ± 2 .61[b]
Blubber depth [cm]	0.8 ± 0.1[a]	2.1 ± 0.1[b]	0.5 ± 0.1[a]
% Blubber by volume	13.47 ± 1.01[a]	34.42 ± 1.42[b]	9.38 ± 1.54[a]
Lipid droplet volume density (%)	72.36 ± 6.22[a]	91.03 ± 2.16[a]	54.40 ± 5.75[b]
% Maternal mass at birth[*]	7%	11%	5%
Nursing period [days][¥]	10 – 12 days	3 – 5 days	35 – 49 days
Pup growth rate [kg day^{-1}][§]	2.3 ± 0.11	5.9 – 7.1	2.0 ± 0.10

Letters in superscript indicate significant differences among mean values ($p < 0.05$) for which there was an effect of species.

[*] From Anderson and Fedak 1987

[¥] Values for harp seals: Kovacs et al. 1991; hooded seal: Lydersen et al. 1997, Bowen et al. 1985, Weddell seals: Teadman and Bryden 1979, Hill 1987

[§] Values for harp seals: Kovacs et al. 1991; hooded seals: Lydersen et al. 1997; Weddell seals: Hill 1987.

Table 1.3: Thermal conductivity (W m^{-1} $°C^{-1}$) of fur including skin, blubber, and sculp (mean ± SEM) in harp, hooded, and Weddell seals. There were no significant differences in conductivity among the species for any tissue ($p > 0.05$).

	Harp seal	**Hooded seal**	**Weddell seal**
Thermal conductivity [W m^{-1} $°C^{-1}$]			
Fur + skin	0.1489 ± 0.0172	0.0892 ± 0.0182	0.1081 ± 0.0176
Blubber	0.2104 ± 0.0633	0.2240 ± 0.0269	0.1785 ± 0.0327
Sculp	0.1567 ± 0.0257	0.1369 ± 0.0120	0.1136 ± 0.0160

Table 1.4: Muscle and brown adipose tissue enzyme activity. Cytochrome c oxidase (COX), citrate synthase (CS), and β- hydroxyacyl CoA dehydrogenase (HOAD) (μmol min^{-1} g-wet $weight^{-1}$) activity and stereological measurement in tissues of harp, hooded, and Weddell seal neonates (mean ± SEM).

		Harp seal	Hooded seal	Weddell seal†
Tissue				
Muscle	Enzyme activity/FMR			
	CS	0.09 ± 0.005[a]	0.09 ± 0.004[a]	0.16 ± 0.028[b]
	COX	0.02 ± 0.001	0.004 ± 0.002	0.02 ± 0.005
	HOAD	0.11 ± 0.004[a]	0.11 ± 0.010[a]	0.93 ± 0.136[b]
	Enzyme activity			
	[μmol min^{-1} g-wet $weight^{-1}$]			
	CS	49.68 ± 3.26[a]	48.57 ± 6.41[a]	23.76 ± 6.28[b]
	COX	6.25 ± 1.27	4.99 ± 0.83	4.40 ± 1.08
	HOAD	70.61 ± 3.04	72.26 ± 5.94	130.01 ± 36.68
Brown adipose tissue	Enzyme activity			
	[μmol min^{-1} g-wet $weight^{-1}$]			
	CS	**102.23 ± 15.75**	**18.17 ± 2.52**	
	COX	**15.47 ± 3.17**	5.67 ± 1.18	
	HOAD	**193.15 ± 31.44**	**31.39 ± 3.63**	
	% Lipid droplet volume density	79.83 ± 3.37	86.61 ± 4.70	
	% Mitochondrial volume density	14.03 ± 2.02	1.40 ± 0.01*	

Letters indicate significant differences in mean values among species ($p < 0.05$). Values in bold indicate significant differences ($p < 0.05$) within species between muscle and brown adipose tissue enzyme activity. * indicates a significant difference ($p < 0.05$) between harp and hooded seal brown adipose tissue mitochondrial density. No brown adipose tissue was found in neonatal Weddell seals. †Weddell seal enzyme values from Kanatous et al. 2008.

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Chapter 2: Shifts in thermoregulatory strategy during ontogeny in harp seals (*Pagophilus groenlandicus*)[1]

Abstract

Heat balance can be difficult for young and/or small animals in polar regions because environmental conditions in combination with small body size or physiological immaturity can increase heat loss. We investigated how thermoregulatory patterns change with ontogeny in 5 age classes of harp seal (*Pagophilus groenlandicus*) from birth to post-molt to further understand the timing of thermoregulatory development in relation to their potential vulnerability to ongoing fluctuations in the extent and stability of Arctic pack ice. We measured changes in the amount, conductivity, and resistance of the seal pups' insulative layers (blubber and fur), the potential for endogenous heat-generation by shivering (muscle enzyme activity), and nonshivering thermogenesis (NST; brown adipose tissue (BAT) uncoupling protein 1 (UCP1) expression and mitochondrial density). There was no significant difference in blubber conductivity among age classes, though the amount of blubber insulation significantly increased from birth to weaning. Pelage conductivity was low (0.12 ± 0.01 W m^{-1} $°C^{-1}$) except in 9-day old pups (0.40 ± 0.08 W m^{-1} $°C^{-1}$); the significantly higher conductivity may signal the beginning of the molt, and this age group may be the most vulnerable to early water entry. Citrate synthase activity significantly increased (49.68 ± 3.26 to 75.08 ± 3.52 μmol min^{-1} g wet $weight^{-1}$) in the muscle; however, it is unlikely that increasing a single enzyme greatly impacts heat generation. BAT of younger pups contained UCP1, though expression and mitochondrial density quickly declined, and the ability of pups to produce heat via NST was lost by weaning. While total thermal resistance did not differ, neonatal and early nursing animals gained the majority of their thermal resistance from lanugo ($82.5 \pm 0.03\%$); however, lanugo is not insulative when wet, and NST may be important to maintain euthermia and dry the coat if early immersion in water occurs. By late nursing, blubber seems sufficient as insulation ($75.87 \pm 0.01\%$ of resistance after 4 weeks), but high

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conductivity of fur may be responsible for retention of UCP1 expression. Weaned animals rely on blubber insulation, and no longer need NST, as wetted fur is no longer a threat to euthermia.

Key words: brown adipose tissue, blubber, lanugo, phocid, thermogenesis, thermal conductivity

Abbreviations:

BAT: brown adipose tissue

COX: cytochrome c oxidase

CS: citrate synthase

HOAD: β-hydroxyacyl CoA dehydrogenase

LD: *longissimus dorsi*

LCT: lower critical temperature

MR: metabolic rate

MT: mitochondria

NST: nonshivering thermogenesis

PWF: post weaning fast

RMR: resting metabolic rate

SA:V: surface area to volume ratio

UCP1: uncoupling protein 1

TEM: transmission electron microscope

TNZ: thermal neutral zone

2.1 Introduction

All mammals must balance metabolic heat production and heat loss to maintain thermoregulatory homeostasis (Scholander *et al.* 1950, 1955). Relative to terrestrial mammals, marine mammals face potential elevated rates of heat loss because of the high thermal conductivity and heat capacity of water compared with air (Scholander *et al.* 1950). Adult marine mammals have evolved a suite of morphological and physiological adaptations to counteract elevated heat transfer when immersed (Irving and Hart 1957; Scholander *et al.* 1950). Adults reduce heat loss to the surrounding environment by having a lower surface area to volume ratio

(SA:V) as compared to terrestrial mammals of similar mass (Innes *et al.* 1990), as well as increased amounts of insulation in the form of blubber and/or fur (Ryg *et al.* 1993; Scholander *et al.* 1950). Vasoconstriction of the periphery and countercurrent heat exchangers conserve heat in the water; conversely, vasodilation in conjunction with countercurrent heat exchangers can dissipate heat when individuals are highly active or hauled out (resting on a terrestrial substrate; Irving and Hart 1957; Scholander *et al.* 1955). As in other endotherms, marine mammals can increase heat production by raising their metabolism and/or initiating shivering thermogenesis. However, metabolic heat production is energetically expensive, and the broad thermal neutral zones (TNZ) of most adult marine mammals indicate these mechanisms are rarely required (Gallivan and Ronald 1979; Hart and Irving 1959; Lavigne *et al.* 1986).

The thermoregulatory challenges faced by young marine mammals differ from those of adults because of habitat, use patterns, and physiological differences (Dunkin *et al.* 2005; Liwanag *et al.* 2009; Noren *et al.* 2003). Most phocids are born on land or ice and do not face the thermoregulatory challenges of an aquatic lifestyle until they begin independent foraging at some point after weaning. However, even when on land, the smaller size and greater SA:V (Blix and Steen 1979), and poor vasocontrol (Lapierre *et al.* 2004) in young pups result in a greater potential for heat loss to the environment than experienced by adults (Blix and Steen 1979). In addition, most phocid pups are born without a thick subcutaneous blubber layer and instead rely on their lanugo, or natal pelage. When dry, lanugo is a more effective insulator than an equivalent thickness of blubber and is also lightweight, so small pups are able to carry a thicker fur layer than they could for blubber (Ryg et al. 1993). However, lanugo loses its insulative capacity when wet and pups must replace it with blubber before beginning foraging activities (Kvadsheim and Aarseth 2002). In contrast, blubber is an internal insulator and retains its insulative capacity regardless of ambient environmental conditions (Kvadsheim and Aarseth 2002; Liwanag *et al.* 2012b). Pups must develop thermoregulatory capabilities of 'aquatic' adults from a 'terrestrial' starting point in a relatively short period (days to weeks), because prolonged immersion during early independent foraging without the physiological capabilities to defend against higher rates of heat loss may energetically compromise young animals (Liwanag *et al.* 2009).

Despite the fact that fur can provide better insulation than blubber, even a thick lanugo coat may not be sufficient insulation to maintain euthermia in polar environments where animals

regularly face gradients of > 40 °C between core and ambient temperatures (Blix and Steen 1979; Grav *et al.* 1974; Øritsland and Ronald 1978). When insulation is no longer sufficient, nonshivering thermogenesis (NST) or shivering thermogenesis may be required to provide the additional heat needed for homeothermy, particularly if the lanugo becomes wet. While NST may provide a mechanism for core warming and for drying saturated lanugo, the use of NST for these purposes results in a substantial increase in resting metabolic rate (RMR) (Cannon and Nedergaard 2008). As the blubber layer develops, similar environmental conditions are much less likely to require NST (Davydov and Makarov 1964; Liwanag *et al.* 2012b). As pups mature, switching insulation from lanugo to blubber is likely essential early survival, especially in the event of early ice break up and immersion, and to reduce thermoregulatory costs once weaned pups foraging.

Harp seals are born on pack ice with a very thin blubber layer and a thick lanugo coat. During the 12-day-nursing period, pups gain mass at a rate of 2–2.5 kg d^{-1}, most of which is deposited as blubber (Kovacs and Lavigne 1986; Oftedal *et al.* 1989; Stewart and Lavigne 1980; Worthy 1991). While early work that located apparent brown adipose tissue (BAT) deposits suggested neonatal harp seals are capable of NST, the presence of uncoupling protein 1 (UCP1) has yet to be confirmed (Blix *et al.* 1979; Grav *et al.* 1974). The utility of BAT would likely be short lived because by weaning, pups have developed a thick (> 5 cm) blubber layer and have begun to molt their lanugo coat, and therefore have functional insulation upon immersion. Pups then remain hauled out 3–4 weeks for the duration of the postweaning fast (PWF) before beginning to forage independently (Stewart and Lavigne 1980; Worthy 1987; Worthy and Lavigne 1987). The loss of lanugo during this period is likely reflective of the increased blubber insulation and decreased SA:V from nursing, the need to prepare for diving, and the probability that lanugo would not be effective insulation as the ice thins and immersion becomes more likely. During the PWF, metabolic costs are met by catabolizing lipid in the blubber layer and protein from the body core, as animals must balance metabolic needs while fasting and the cost of thermoregulation (Worthy and Lavigne 1987). Fatter animals or those that lose weight more slowly, typically fast for longer periods (Noren *et al.* 2003; Noren *et al.* 2008), potentially reducing protein catabolism, and allowing more time for physiological development of the oxygen stores, muscle structure, and biochemistry necessary for sustained diving and foraging (Burns *et al.* 2007; Lestyk *et al.* 2009; Noren *et al.* 2008).

This study examines how the thermoregulatory strategy of harp seal pups changes as pups grow and prepare for independent foraging. The morphological transition from lanugo to blubber in harp seals, like other phocids, has been well documented (Kvadsheim and Aarseth 2002; Oftedal *et al.* 1996; Worthy 1991). However, we were interested in quantifying insulative properties of the fur and blubber during ontogeny, examining potential capacity of heat production through NST and shivering, and determining if changes in insulation were correlated with changes in the thermogenic capacity. We hypothesized that neonatal pups with lanugo coats require or possess additional heat generating mechanisms like NST, but as pups grow and the blubber layer thickens to provide equivalent internal insulation, additional heat generating mechanisms should no longer be necessary. Understanding the timing of thermoregulatory development in harp seal pups is important for understanding their potential vulnerability to ongoing changes in the extent and stability of Arctic pack ice (Post *et al.* 2013). Declines in pack ice and increases in storm events in the Arctic may increase the chance young pups enter the water earlier or more frequently than in the past (Friedlander *et al.* 2010; Hansen *et al.* 2013; Post *et al.* 2013). If these changes occur before pups have developed insulation that is capable of dealing with the increased thermal capacity of water, they may reduce pup survival in the days and weeks post weaning.

2.2 Materials and Methods

2.2.1 Animals and sample collection

To assess potential changes in thermoregulatory strategies with development, neonatal (within ~24 hr of birth; n = 6), newly weaned (~12–15 days old; n = 5), and post-weaned (~21 days old; n = 5) harp seal pups, and 4 adult harp seal females were hand-captured in March 2008 in the Gulf of St. Lawrence, Canada (47°36' N, 62°13' W). Additionally, neonatal (n = 4), early nursing (~4 days old; n = 3), and late nursing (~9 days old; n = 4) animals were hand-captured in March 2011 in the "West Ice" off Greenland (72°24' N, 14°15' W). Together, these two sampling periods represent 5 developmental age classes from birth to late weaning. Animals were aged following Stewart and Lavigne (1980), sacrificed using methods approved for scientific harvest in Canada or Norway, and samples were imported to the United States under NMFS Permit #782-1694-02 (Canada) and 15510 (Norway). The University of Alaska

Anchorage Animal Care and Use Committee approved all sampling protocols (#Burns2005 and #149278-1).

2.2.2 Morphometrics and body condition

To assess changes in body condition and blubber thickness, all pups (n = 27) were weighed to the nearest 0.5 kg using a spring scale. Post-mortem measurement of straight length was taken with a tape measure and blubber thickness was measured using a ruler to the nearest 0.1 cm at 4 locations (neck, axillary, umbilicus, and pelvis) along the dorsal and ventral axis of the body. Mean blubber depth was calculated as the average of all measurements taken from an individual. The mass:length ratio was calculated for all animals, and a greater mass:length ratio was assumed to be indicative of better condition. For the pups collected in Greenland (n = 11), girth and curvilinear length measurements were taken between each blubber depth measurement location, and surface area, body volume, and % blubber by volume calculated using equations published by Gales and Burton (1987). Lean tissue volume was calculated as the difference between total volume and blubber volume.

2.2.3 Assessment of insulation: conductivity, and heat flux

We assessed the quality of insulation provided by blubber and fur by measuring the thermal conductivity (k) of the pelt according to the methods outlined by Kvadsheim *et al.* (1994), Dunkin *et al.* (2005) and Liwanag *et al.* (2012a, b). Sculp samples (full blubber thickness + skin + fur) were collected from all 5 pup age classes and the adults (n = 31 total) within 30 min post-mortem from the mid-trunk region and stored at -20 °C. Thermal conductivity of the sculp, blubber (alone), and pelt (fur with skin) was measured on square (~10 cm × 10 cm) samples using the 'standard materials method' described by Dunkin *et al.* (2005) and Liwanag *et al.* (2012a,b). An elastomer (Plastisol vinyl, Carolina Biological Supply, Burlington, NC, USA) was used as the standard material ($k = 0.109 \pm 0.0006$ W m^{-1} $°C^{-1}$), and was placed in series with the sculp sample. Three thickness measurements to the nearest 0.01 mm from each side of the sample were taken of the blubber, skin, and dry fur using digital calipers (ABSO-LUTE Digimatic Caliper Series 500, Mitutoyo, Aurora, IL, USA); mean values were used for calculations. Thermal conductivity was calculated across the sculp, blubber, and pelt, using the Fourier equation (Kreith 1958):

(Eq. 2.1) $$k = H \times L / A \times \Delta T$$

where k is conductivity (W m^{-1} $°C^{-1}$), H is heat transfer (J s^{-1}), L is the thickness of the material (m), A is the area (m^2) through which the heat is moving, and ΔT is the temperature differential (°C) across the material. As heat transfer is assumed to be equal across the standard material and sample, the equations were set equal and solved for the thermal conductivity of the sample. To account for changes in insulation due to changes in the thickness of blubber and fur with development, thermal resistance (R; m^2 °C W^{-1}), was calculated for the sculp, blubber, and pelt using the equation:

$$R = L / k \qquad \text{(Eq. 2.2)}$$

The rate of heat loss or gain from the environment was measured at 6 locations along the body (ears, neck, axillary, umbilicus, pelvis, and ankles) for 11 live pups from Greenland, using a heat flux sensor (Thermonetics Inc, San Diego, CA, USA) attached to a digital multimeter. All measurements were taken within 12 hrs of capture and while animals were resting in outdoor enclosures aboard the ship. Ambient air temperature was measured during all heat flux measurement periods and averaged -1.0 ± 0.5 °C. Pups were protected from wind by their enclosures. After completion of heat flux measurements, animals were sacrificed and samples collected as described in *2.2.1, 2.2.4* and *2.2.5*. Heat flux measurements were converted to W m^{-2} using the calibration factor provided by the manufacturer. Mean heat flux was calculated as the mean of the heat flux values from all body locations.

2.2.4 Assessment of the potential for metabolic heat production

To assess the capacity for metabolic heat production by the *longissimus dorsi* (LD) muscle through either shivering thermogenesis or futile cycling, we measured the activity of three metabolic enzymes that play important roles in providing reducing substrates for heat production: citrate synthase (CS) as an estimate of TCA cycle activity, β-hydroxyacyl CoA dehydrogenase (HOAD) as a measure of lipid β-oxidation, and cytochrome c oxidase (COX) as a measure of electron transport chain activity. We measured all enzyme activities under substrate saturating conditions following previously published protocols (Kanatous *et al.* 2008; Prewitt *et al.* 2010). Post-mortem samples of LD (100 mg) were immediately frozen in liquid nitrogen upon collection and stored at -80°C until assayed. Samples were homogenized at 0°C in buffer (1:20 wt:vol) containing phosphate buffered saline, 1% Tween 20, 20% glycerol, and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) and centrifuged at 10,000 g

for 10 min at 4°C. The supernatant was used for the enzyme assays, which were run in a Molecular Devices SpectraMax 340 microplate reader (Sunnyvale, CA, USA) held at body temperature (37 °C). Assay conditions were as follows: CS (EC 4.1.3.7): 50 mmol l^{-1} imidazole, 0.25 mmol l^{-1} 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 0.4 mmol l^{-1} acetyl CoA and 0.5 mmol l^{-1} oxaloacetate, pH 7.5 at 37° C; ΔA_{412}, $\in_{412}$=13.6. HOAD (EC 1.1.1.35): 50 mmol l^{-1} imidazole, 1 mmol l^{-1} EDTA, 0.1 mmol l^{-1} acetoacetyl CoA, and 0.15 mmol l^{-1} NADH, pH 7.0 at 37° C; ΔA_{340}, $\in_{340}$=6.22. COX: 0.1 mmol l^{-1} DTT, 0.22 mmol l^{-1} ferrocytochrome-c, 10 mmol l^{-1} Tris-HCl pH 7.0 with 120 mmol l^{-1} KCl; ΔA_{550} $\in_{550}$=21.84 (Sigma Aldrich #CYTOCOX100). Samples were run in triplicate; values were only accepted if replicate coefficient of variations (CVs) < 10 %. Activity values for the entire assay plate were rejected and rerun if values for a control muscle with known enzyme activity fell outside a previously determined normal range (Prewitt et al. 2010). Specific enzyme activities (IU g^{-1} wet tissue mass) were calculated from the change in absorbance at the linear slope of the assay.

2.2.5 Assessment of potential for nonshivering thermogenesis

To determine if harp seals were capable of NST we examined animals for the presence of BAT in previously described locations: the *venus plexus* of the neck, subcutaneously in the blubber, and superior to the kidneys (Blix *et al.* 1979; Grav *et al.* 1974). We collected samples of a tissue that matched the gross morphological descriptions of BAT (Afzelius 1970; Grav *et al.* 1974) from the *venus plexus* of the neck from all 5 age classes of pups (n = 27). No other locations had BAT-like tissue. All tissue samples were stored in Whirlpaks® at -20 °C for up to 2 weeks before being transferred to -80°C until analysis. Blubber and muscle (LD) samples were also collected for use as negative controls in Western blot analysis.

Western blot analyses were performed on 12% SDS-PAGE gels with tissue homogenates of the BAT-like tissue to determine if UCP1 was expressed from all age classes sampled (n = 27). Samples were homogenized in the same buffer used for enzyme assays and centrifuged at 10,000 g for 10 min at 4 °C. Total protein content of the homogenate was determined using Pierce Coomassie Blue 'The Better Bradford' Total Protein Assay (Pierce Chemicals, Rockford, IL, USA). Thirty micrograms protein per sample were mixed with loading dye, loaded onto 12% SDS - PAGE gels, run at 100 V for 1 hr, and transferred onto nitrocellulose membrane. Membranes were then stained with Ponceau S Solution (0.2% v/v in 5% acetic acid; Alfa Aesar,

Ward Hill, MA, USA) to ensure proper protein transfer. Western blot development was done on a SNAP i.d. (EMD Millipore, Billerica, MA, USA). The primary antibody (rabbit anti-UCP1, IgG, 1:3000; #ab10983, Abcam, Cambridge, MA, USA) was detected using an Alexa Fluor 680 goat anti-rabbit (IgG, 1:5000) secondary antibody (Invitrogen, Carlsbad, CA, USA). Additionally, β-Actin (rabbit anti- β-Actin, IgG, 1:3000, #ab8227, Abcam, Cambridge, MA, USA) was detected on the same membranes, to ensure equal protein loading across samples and gels. Protein bands were visualized using a LI-COR Odyssey imaging system (LI-COR, Lincoln, NE, USA) and band intensity was quantified using a digital analysis program (Image J, Schneider *et al.* 2012). Samples were run in triplicate, and expression of UCP1 and β-Actin were calculated for each individual. UCP1 expression relative to β-Actin expression was calculated for each individual and averaged for each age class. Arctic ground squirrel (*Urocitellus parryii*) BAT was used as a positive control for antibody reactivity to UCP1. We tested for cross-reactivity of the antibody with other UCPs by performing Western blot analyses using the UCP1 antibody on muscle and blubber tissues. Peptide competition assays were also performed to ensure binding was not due to antibody cross-reactivity. In this assay, UCP1 antibody (1:1500) was pre-incubated with UCP1 peptide (1:60; #ab24282, Abcam, Cambridge, MA, USA) for 60 min at 37°C. Incubation and development proceeded as above using the primary antibody-peptide mix in place of the primary antibody. Peptide competition resulted in complete inhibition of antibody activity, indicating the protein bound by the UCP1 antibody in the Western blot was UCP1 and not antibody cross-reactivity.

The mitochondrial (MT) volume density in BAT was measured to assess tissue ultrastructure and potential for thermogenesis in the harp seal samples collected in Greenland, representing pups during the nursing period (n = 11). In the field, 15 mg sub-samples of BAT-like tissue were immediately fixed in a 2% gluteraldehyde- 2% paraformaldehyde, 0.1 M sodium cacodylate buffer (pH 7.4) and stored at 4 °C. Subsequently, at the Electron Microscopy Lab at the University of Maine (Orono, ME), samples were cut into 1 mm blocks, and rinsed 3x and held overnight in 0.1 M cacodylate buffer (pH 7.4). Samples were then post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hr while on ice, rinsed in diH_2O, and dehydrated with increasing concentrations of acetone (50–100%). Samples were infiltrated with 50/50 mix of 100% acetone and epon-araldite resin overnight, moved to fresh resin under vacuum (2x 30 min) the following day, embedded in fresh epon-araldite resin, and cured for 48 hrs at 65 °C. Thin

sections (2 μm) were cut on a Leica UC6 Ultramicrotome (Leica Biosystems, Buffalo Grove, IL, USA) and placed onto G200 copper grids (Electron Microscopy Sciences, Hatfield, PA, USA). Grids were counter-stained with 1% uranyl acetate in water for 20 min followed by 0.5% lead citrate in water for 4 min. Sections were photographed on a JEOL 1200 Transmission Electron Microscope (TEM; JEOL, Peabody, MA, USA) in the Advanced Instrument Lab at the University of Alaska Fairbanks. Carbon grating replica calibration was performed on the TEM to confirm magnification was within 5% of nominal magnification. Digital images were captured at 3000×. Twenty images were taken per sample for analysis. The volume fraction of mitochondria was estimated using standard point counting procedures (Weibel 1979) modified for digital photography (Watson *et al.* 2007). Adobe Photoshop CS5 (Adobe Systems Inc., San Jose, CA, USA) with the 'grid' feature enabled was used to generate a grid of appropriate point density for each tissue based on the relative size of the mitochondria (Weibel 1979). All points falling on mitochondria were counted. The relative standard errors (RSE) of the volume density of each sample were calculated by pooling counts from all images for a sample, and applying the RSE equation for binomial counts (Mathieu *et al.*1981). RSE was 7.69 ± 1.31% for all samples.

We evaluated the potential for metabolic flux through mitochondria in BAT from all 5 age classes of pups sampled (n = 27), by measuring the aerobic enzyme activities (μmol ml^{-1} g^{-1} wet tissue) of CS, HOAD, and COX using the same protocols used for LD muscle described in Section *2.2.4*.

2.2.6 Statistical analysis

Data were tested for normality and transformations were used as necessary. No data points were identified as outliers (mean ± 2 SD), so all data points were retained. One-way ANOVAs and Bonferroni post-hoc tests were used to determine differences among age classes, and significance was considered at the 95% level ($P < 0.05$). Age-related changes in mean and location-specific heat flux measurements were analyzed using a non-parametric Kruskal-Wallis test. To determine the correlation between insulation and NST in nursing pups, step-wise linear regression models were used. In this analysis, mean blubber depth, fur thickness, thermal resistance of blubber, and mass:length ratio were used as metrics of insulation and condition, and UCP1 expression was used as a metric of NST potential. UCP1 data were transformed using a

Box-Cox power transformation with lambda = 0.2 prior to regression analysis. All analyses were completed in SPSS Software (v 21, IBM, Armonk, NY, USA).

2.3 Results

2.3.1 Animal growth and changes in insulation

Mass, length, and mass:length ratio of pups increased from birth through the nursing period (mass: $F_{4,20} = 97.32$, $P < 0.001$; length: $F_{4,20} = 6.29$, $P = 0.002$; mass/length: $F_{4,22} = 75.67$, $P < 0.001$; Table 1). As pups increased in mass, their body volume and surface area also increased (body volume: $F_{2,8} = 17.75$, $P = 0.001$; surface area: $F_{2,8} = 20.01$, $P = 0.001$), primarily due to an increase in blubber from 13.75 ± 1.03% in neonates to 43.50 ± 1.84% in newly weaned pups. However, because pup volume increased more rapidly than surface area, the SA:V ratio significantly decreased over the nursing period ($F_{2,8} = 18.42$, $P = 0.001$, Table 1). Body mass, but not length, decreased during the post-weaning fast, likely causing a small secondary increase in SA:V, but this was not measured in this study because no weaned pups were sampled in Greenland.

The increase in relative % blubber with age was paralleled by the increase in blubber thickness at all locations along the body (neck $F_{4,20} = 45.46$, $P < 0.001$; axillary $F_{4,20} = 80.03$, $P < 0.001$; umbilicus $F_{4,20} = 95.26$, $P < 0.001$; pelvis $F_{4,20} = 58.67$, $P < 0.001$; Figure 1), and mean blubber thickness quintupled between birth and weaning ($F_{4,22} = 104.45$, $P < 0.001$; Table 1). Fur thickness averaged 2.410 ± 0.020 cm in neonates and did not change during the nursing period. Thickness declined as pups began to molt; in weaned pups, fur thickness decreased by nine fold ($F_{4,23} = 26.09$, $P < 0.001$; Table 1).

The mean conductivity of harp seal blubber was 0.2100 ± 0.0183 W m^{-1} $°C^{-1}$. There was no significant difference in blubber conductivity among the 5 age classes of pups or between the pups and adults (Table 2), indicating the material properties of blubber did not change with ontogeny. However, the material properties of the pelt did change with age: pelt conductivity was highest in pups late in the nursing period just before the lanugo was shed ($F_{5,27} = 12.35$, $P < 0.001$; Table 2). As a result, the thermal conductivity of the sculp (blubber + fur + skin) was highest in late-nursing pups compared to other pups and adults ($F_{5,27} = 4.71$, $P = 0.005$). Once

weaned, sculp conductivity declined, largely due to a 67% decrease in the conductivity of the pelt component (Table 2).

While there were no significant changes in the overall thermal resistance of the sculp with age class or between pups and adults, there were significant differences in the relative contribution of blubber and fur to the overall thermal resistance. As blubber layer thickness increased, the proportion of the overall thermal resistance due to blubber also increased ($F_{4,27}$ = 21.09, $P < 0.001$). Similarly, as fur thickness decreased, there was a significant decrease in the resistance of the pelt ($F_{5,27}$ = 11.42, $P = 0.001$; Figure 2). In weaned pups, both the overall thermal resistance and the relative contributions of fur and blubber were similar to adult values (Figure 2). Remarkably, for the heat flux measurements on live pups, there was little effect of age class or body location on overall heat flux rates in nursing animals and mean heat flux was not significantly different among age classes (Figure 3), with the exception of slightly higher rates of heat flux in neonatal animals.

2.3.2 Potential for metabolic heat production

The potential for metabolic heat production through shivering or futile cycling, as indicated by enzyme activity in the LD muscle, generally increased with age class, although these increases were not always statistically significant, nor the patterns linear (Table 3). For example, COX activity was significantly higher in both neonates and weaned pups as compared to nursing pups ($F_{4,20}$ = 3.91, $P = 0.017$). In contrast, CS activity was significantly lower in all nursing pups as compared to all weaned pups ($F_{4,20}$ = 12.01, $P < 0.001$), and HOAD activity did not differ significantly among age classes ($F_{4, 20}$ = 2.85, $P = 0.051$).

2.3.3 Potential for nonshivering thermogenesis

BAT-like tissue was found in, and collected from, the dorsal intrascapular region near the *venus plexus* of pups of all age classes. BAT was not found above subcutaneously or superior to the kidneys. Expression of UCP1 (relative to β-Actin) in this tissue was highest in neonates and declined precipitously with age class, such that it could not be detected in any weaned pups ($F_{4,23}$ = 37.12, $P < 0.001$; Figure 4A-D). While the BAT of neonatal harp seals had multilocular lipid droplets and high MT density, as UCP1 expression declined with age, MT density in BAT decreased 86% by weaning, from a high of 13.88 ± 2.00% in neonates to 4.50 ± 2.80% by early nursing, and 1.95 ± 0.87% in late weaned pups ($F_{2,10}$ = 11.00, $P < 0.003$; Figure 5A-C).

Concomitant with the decline in mitochondrial density, enzyme activity (COX, CS, HOAD) in BAT also declined over this period, and by early weaning COX activity was approximately 20% of activity in neonates, and CS and HOAD declined by 50% (Table 3; COX: $F_{4,20} = 5.80$, $P = 0.003$; CS: $F_{4,20} = 10.07$, $P < 0.001$; HOAD: $F_{4,20} = 7.00$, $P = 0.001$). The decline in CS and COX (82% and 85% decline by weaning, respectively) closely match the 86% decline in MT density. Combined, these further suggest the ability of BAT to produce heat via NST was functionally absent by early weaning. Step wise regression analysis revealed that in nursing animals, UCP1 expression was negatively correlated with mean blubber depth ($F_{1,16} = 14.603$, $P = 0.002$ $R^2 = 0.493$; Figure 6); UCP1 was not expressed once blubber depths were greater than 3.2 cm, and not expressed in any weaned pup (Figure 6). Mean blubber depth was significant ($t = -3.821$, $P = 0.002$); no other variables were included in the regression model because of multicolinearity, and when included individually with mean blubber thickness, they did not improve model fit.

2.4 Discussion

The results of this research show that neonatal harp seals have equivalent thermal resistance as older, fatter pups. But because this insulation largely comes from a wettable lanugo coat, they possess the potential for additional thermogenesis by NST through the expression of UCP1 in BAT. As animals progress through the nursing period and gain substantial blubber volume, this forms their main insulative layer and source of thermal resistance. As older pups no longer rely on a wettable coat for insulation, additional thermogenesis is not necessary, and the ability for NST is lost. The potential for shivering thermogenesis does not appear to change across ontogeny, and is thus not an important source of heat generation in smaller animals.

The nursing and early developmental period is critical for animals, and for phocids this is the time when all energy reserves to fuel the PWF and initial foraging activities are accumulated (Oftedal *et al.* 1989; Stewart and Lavigne 1980; Noren *et al.* 2008). Pups that can maximally reduce metabolic expenditure during the nursing period are able to allocate the largest fraction of milk energy to blubber, and thus enter the PWF with larger energy reserves to withstand PWF and initiate independent foraging. Maintaining thermoregulatory homeostasis through insulative mechanisms and reducing reliance on excess heat production via NST or shivering is essential to reducing metabolic costs. By refraining from swimming and diving throughout the nursing period (Kovacs and Lavigne 1986) harp seals decrease the chance the lanugo becomes wet,

decreasing the need for NST, and their thermoregulatory strategy reflects of this behavior. Unlike species that enter the water soon after birth such as hooded seals (*Cystophora cristata*; Oftedal *et al.* 1991), neonatal harp seals rely on their lightweight lanugo for insulation, and young harp seals do not have sufficient body size to support the amount of blubber necessary to provide adequate insulation in air (Liwanag *et al.* 2012a; Ryg 1993). Based on our thermal resistance data, ~4 cm of blubber provides equivalent insulation as ~2.4 cm of lanugo. If pups were born with equivalent blubber insulation instead of lanugo, they would be over 75% blubber, which is quite unrealistic. However, should their lanugo become saturated with water, their high SA:V and thin subcutaneous blubber layer may lead to high rates of heat loss and therefore necessitate additional heat generation in the form of NST. As pups grow and deposit blubber reserves during the nursing period, their SA:V declines and primary insulation shifts from lanugo to blubber, and the potential need for NST is lost. Thus it appears that the large increase in blubber (volume and % total body volume) during the nursing period is essential for both the accumulation of energy reserves to fuel the PWF, and the formation of a thermal barrier that is effective when wet (Liwanag *et al.* 2012b; Noren *et al.* 2008). Indeed, fasting harp seal pups (post-weaning) have the same MR whether they are fasting in water or air (Worthy and Lavigne 1987).

For species that are born with lanugo, the thick fur allows the outer skin to be maintained close to core body temperature while the blubber develops, and even once the blubber is thick, the lanugo likely facilitates maintaining warmer skin temperatures during the post natal molt. This is a critical period when a juvenile pelage is grown, and replaces the lanugo fur, which is shed. As the skin must be kept warm during molting (Ling *et al.* 1970), beginning the molt while lanugo is still present and naturally facilitates warm skin temperatures may be beneficial in lowering metabolic costs associated with the postnatal molt. We observed initial growth of juvenile pelage in 3-day old animals (Liwanag and Pearson unpublished data). Additionally, in this study, though late nursing animals' coats were not visually different from younger animals, pups had much higher pelt conductivity than all other age classes, and this may signify the true beginning of the molt before the adult hair has fully grown.

In adult marine mammals, inter-species comparisons indicate the thermal conductivity of blubber is inversely proportional to the blubber % lipid (Liwanag *et al.* 2012a, b; Worthy and Edwards 1990). However, this trend is seen across animals of varying health and condition, and

lower conductivity values were reported in animals of poor condition with less than 50% lipid in the blubber (Liwanag *et al.* 2012a,b; Dunkin *et al.* 2005). Dunkin *et al.* (2005) reported no change in conductivity despite increases in blubber lipid content for bottlenose dolphins (*Tursiops truncatus*) during development. The thermal conductivity of harp seal blubber did not change during ontogeny and was not different between pups and adults, but increased blubber thickness resulted in a concomitant increase in the proportion of thermal resistance from blubber as the animals aged. Similar findings in both dolphins and harp seals suggest young animals that are quickly depositing lipid, thus accruing blubber with a high lipid content (Kovacs and Lavigne 1985), have potentially already minimized the conductivity of the blubber. As a result, additional lipid serves only to increase depth and thus resistance of the layer.

The TNZ of harp seal pups is determined by overall conductivity and resistance, but previous studies have only considered the contribution from blubber. Our work suggests, especially in very young pups, the fur is important in determining overall thermal resistance, and low conductivity. While direct measurements of harp seal pup TNZ in air do not exist, model estimates based solely on blubber conductivity measurements predict the lower critical temperature (LCT) of neonates and early nursing animals (blubber depths = 1 cm) to be -1 °C in air, whereas the predicted LCT of weaned and fasting animals (blubber depths = 10 cm) are -59 °C and -85.4 °C (Øritsland and Ronald 1978; Worthy 1991). In the Arctic, average temperatures during the March nursing period range from -20 °C to 10 °C, suggesting that neonatal and nursing pups may face environmental conditions below their LCT. However, the insulative value of lanugo is not included in these models, as models are based the conductivity of blubber alone rather than the sculp. This study showed the conductivity of the sculp is lower than the conductivity of blubber only (Table 1), and it may be that the LCT of young pups is lower than reported by Øritsland and Ronald (1978).

Previous observations of young harp seal pups suggest they only rely on shivering thermogenesis during the first few hours after birth (Blix *et al.* 1979). Our examination of the activity of enzymes involved in muscle contraction and shivering thermogenesis support this conclusion, as activities were not any higher in nursing pups than in older animals. The exception to this was elevated COX in neonates only, which could reflect the observations of Blix *et al.* (1979). These results suggest avoidance of shivering thermogenesis, possibly because heat generation via muscle metabolism would be in conflict with rapid energy deposition during

nursing and energy savings needed in preparation for the PWF. Alternatively, changes in muscle enzyme activity with age may reflect changes in muscle structure as pups develop the low oxygen use rates necessary for diving. The slight increase in enzyme activity post weaning is more likely reflective of muscle development and remodeling that occurs in preparation for an aquatic lifestyle and independent foraging (Burns *et al.* 2007; Lestyk *et al.* 2009; Noren *et al.* 2008) than changes due to thermoregulatory constraints.

While young harp seals apparently do not rely heavily on shivering thermogenesis, they do possess the ability to use NST when ambient temperatures are below their LTC. Neonatal harp seals have BAT that expresses UCP1 and resembles the BAT of other species such as Arctic ground squirrels (*U. parryii*) and Syrian hamsters (*Mesocricetus auratus*) (Afzelius 1970). We did not observe any of the additional BAT deposits described by Blix *et al.* (1979), and Grav *et al.* (1974) and found no other tissue expressing UCP1; thus, it appears in neonatal harp seals the subscapular BAT deposit is the only tissue capable of generating heat via NST. This, in combination with the 86% total decrease in mitochondrial density and accompanying 82-85% decrease enzyme activity, suggests the need for NST declines quickly during the nursing period mirroring the increase in blubber, and by weaning, additional thermogenesis by NST is no longer necessary. The decline in thermogenic activity of BAT at 9 days in this study is later than reported by Blix *et al.* (1979). This also suggests NST serves a dual role in young harp seals: (1) maintaining euthermia when pups are dry but ambient temperature is very low, and (2) warming the body to dry the fur when the pelage becomes wet from storms or immersion. Though, to date, only harp and ringed seals (*Pusa hispida*) are known to express UCP1 (Taugbol 1982), NST is likely an important mechanism for maintaining euthermia for other small species born with lanugo on pack ice such as spotted seals (*Phoca largha*) and ribbon seals (*Histriophoca fasciata*). For polar species born with a thicker blubber layer, such as hooded seals (*C. cristata*), NST may not be necessary; for species born on a more stable substrate such as Weddell seals (*Leptonychotes weddellii*), NST may not be necessary because the threat of early water entry is minimal.

In nursing harp seal pups, UCP1 expression is negatively correlated with the increase in blubber insulation, and expression is not present in pups with a blubber layer thicker than 3.2 - 3.5 cm, or any weaned pup (Figure 6). This suggests pups that are able to accumulate blubber more quickly may rely less extensively on NST and reserve more of the energy acquired during

nursing for PWF. Such linkages between insulation and heat production offer mechanisms by which both maternal efforts and environmental conditions can influence pup growth rates and potential survival. For example, in many species, smaller, less-experienced mothers transfer less energy to their pups during the nursing period, resulting in decreasing pup growth and blubber deposition rates (Iverson *et al.* 1993; Mellish *et al.* 1999; Stewart and Lavigne 1984). Small pups would have higher thermoregulatory costs, and would lose mass and condition at a greater rate during the PWF, ultimately resulting in pups in poor condition entering into the initial foraging period (Kovacs and Lavigne 1986; Mellish *et al.* 1999). Fuel use during the PWF is influenced by body composition at weaning, and preservation of the blubber layer while fasting is particularly important in species that may enter the water, which is reflected in harp seals' use of both muscle and blubber to fuel the PWF (Worthy and Lavigne 1987). Such effects on growth would be magnified during cold or stormy springs. Larger pups of better mothers may be buffered from environmental impacts on pup growth because of higher rates of energy transfer and blubber deposition (Iverson *et al.* 1993; Kovacs and Lavigne 1986; Mellish *et al.* 1999)

Current environmental conditions in the Arctic are warming rapidly, resulting in reduced sea ice stability, depth, and duration (Friedlaender *et al.* 2010; Hansen *et al.* 2013; Post *et al.* 2013; Walsh 2008), and a predicted increase in storm events (Schultz 2013; Vermaire et al. 2013). Harp seals, like other Arctic phocids, are dependent on a stable sea ice substrate during the nursing and PWF periods (Bajzak *et al.* 2011; Friedlaender *et al.* 2010; Moore and Huntington 2008) and poor ice conditions are known to increase pup mortality (Bajzak *et al.* 2011; Ferguson *et al.* 2005; Friedlaender *et al.* 2010; Johnston *et al.* 2012; Kovacs and Lydersen 2008). Our results suggest if pups are forced to enter the water early, nursing harp seals pups would have elevated thermoregulatory costs as compared to weaned pups, because of their lack of blubber. The increased thermoregulatory costs could have negative consequences on their survival (Davydov and Makarov 1964; Smith and Harwood 2001; Worthy 1991). While harp seals gain blubber quickly (Kovacs and Lavigne 1985) and thus have a relatively short period of vulnerability, this may not be the case in species with prolonged development periods and smaller birth size, such as ringed and spotted seals (Ferguson *et al.* 2005; Oftedal *et al.*1996). As the pups of seven of eleven polar phocids rely on lanugo when young, these finding suggest that vulnerability to changes in ice conditions may be widespread. While it may be possible for some species to shift from pupping on pack ice to nearby shorelines, such changes carry other risks

such as predation, disturbance, and disease transmission. Ultimately, more research is needed to understand the mechanisms, costs, and timing associated with development of thermoregulatory strategies in young polar marine mammals and to predict the vulnerability to climate change of different ice-breeding seals during the critical time of early development.

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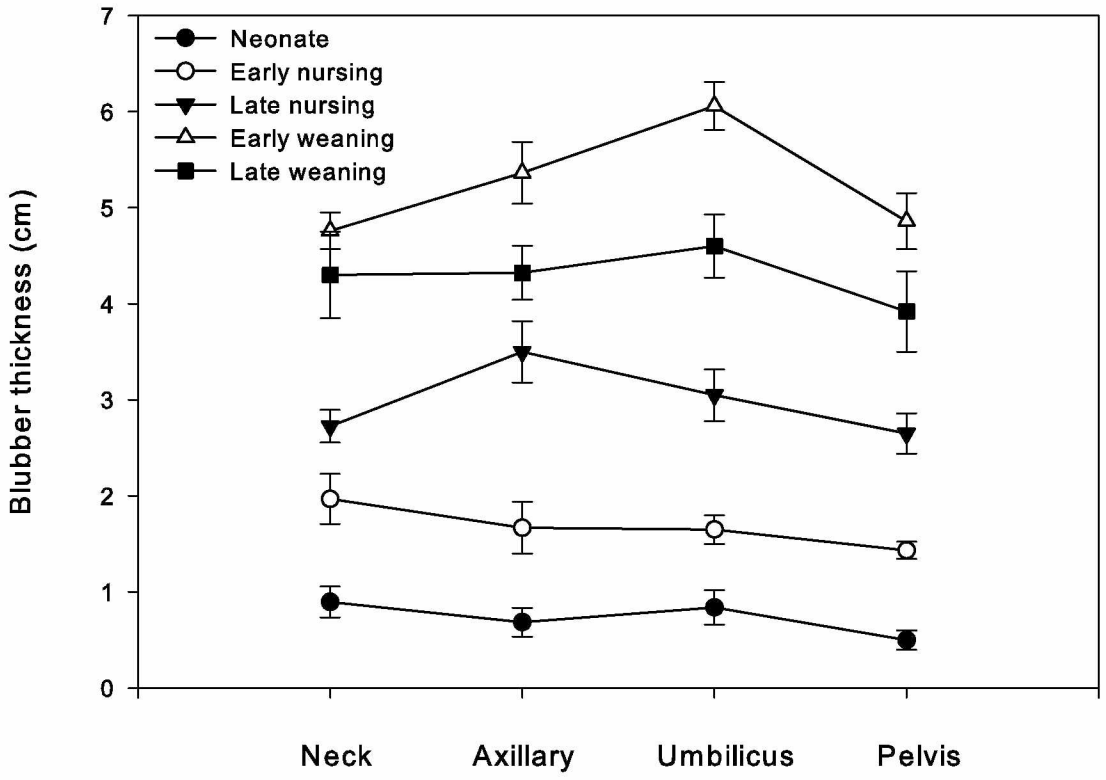

Figure 2.1: Blubber thickness (cm ± SEM) of harp seals. Measurements taken during ontogeny across the body; measurements along the dorsal midline at the neck, axillary, umbilicus, and pelvis. At each location along the body, there was a significant increase in thickness between each age class ($P < 0.05$).

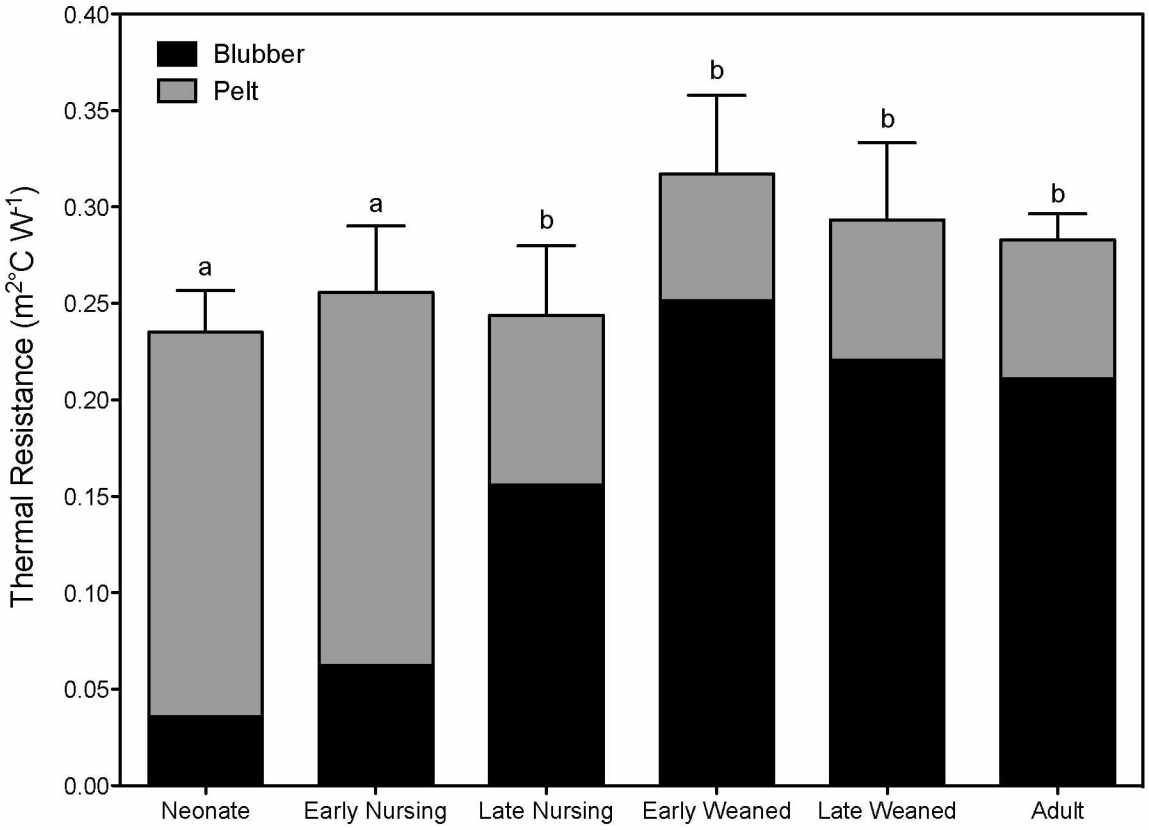

Figure 2.2: Mean (± SEM) total thermal resistance of harp seals. Total thermal resistance is the sum of the thermal resistance of the blubber and pelt. There was a significant increase ($P < 0.05$) in the resistance of blubber with age, and an equivalent decrease in pelt resistance ($P < 0.05$) with age. Because significant changes in the resistance of the blubber and pelt resulted in the same groupings, letters indicate statistically significant differences ($P < 0.05$) between mean values for which there was an effect of age on both the resistance of blubber and pelt.

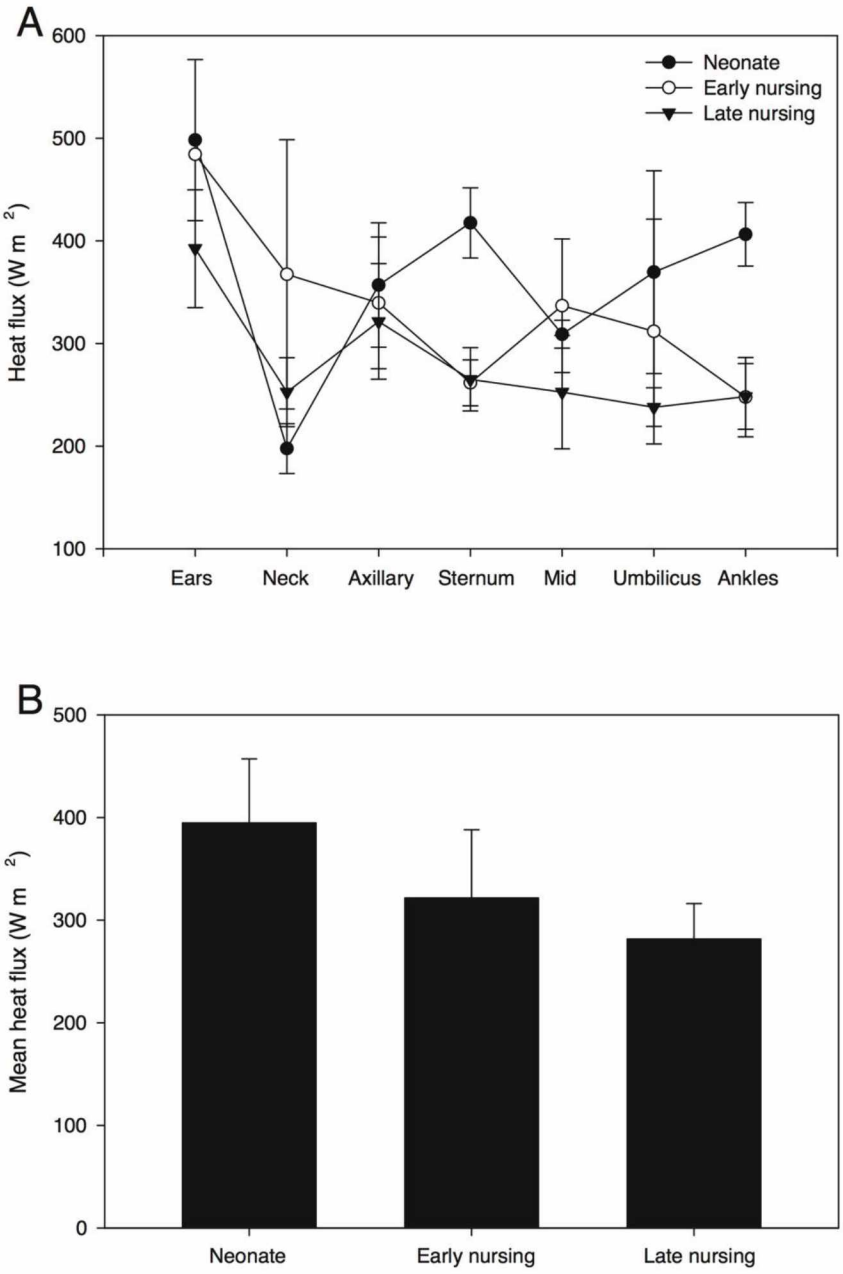

Figure 2.3: (A) Heat flux (± SEM) of nursing harp seals. At 6 dorsal midline locations: ears, neck, axillary, sternum, mid, umbilicus, and ankles. There were no significant differences between age classes in heat flux at any location along the body ($P > 0.05$). (B) Mean heat flux ± SEM, averaged from all dorsal heat flux measurements across the body, did not differ significantly between age classes ($P > 0.05$).

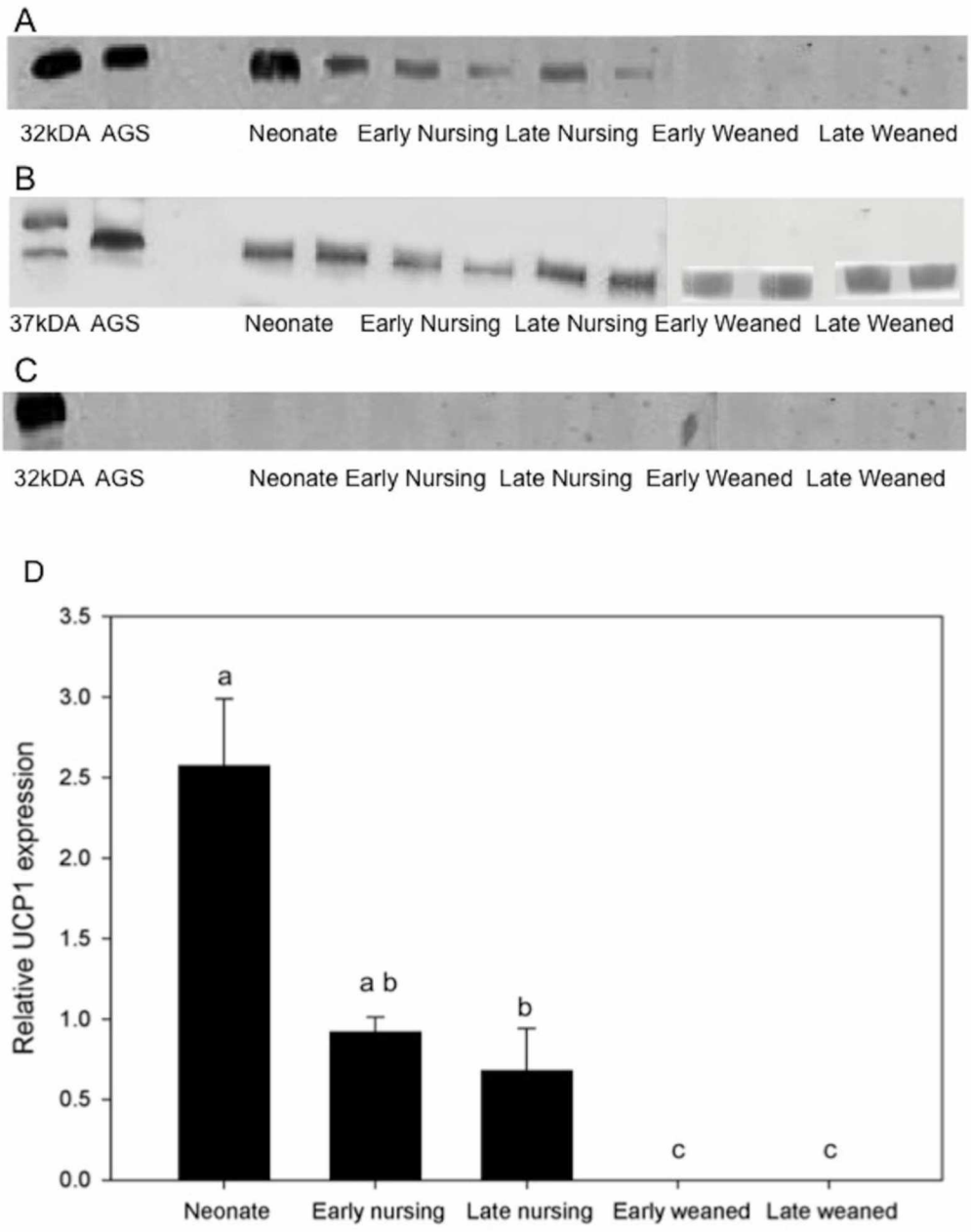

Figure 2.4: (A) Representative Western blot of uncoupling protein 1 expression. Brown adipose tissue from 2 individuals from each of the 5 age classes of harp seal pup. Each band represents an individual pup. (B) Representative Western blot of the loading control, β-Actin, in brown adipose tissue from 2 individuals from each of the 5 age classes of harp seal pup. Each band represents the same pup shown in (A). (C) Complete blockage of antibody binding in the peptide inhibition assay. Each band represents an individual pup, and are the same pups as in (A) and (B). (D) Relative UCP1 protein expression (± SEM) in different age classes of harp seals during ontogeny, as determined by digital analysis of band intensities from Western blots. Letters indicate statistically significant differences ($P < 0.05$) between mean values for which there was an effect of age.

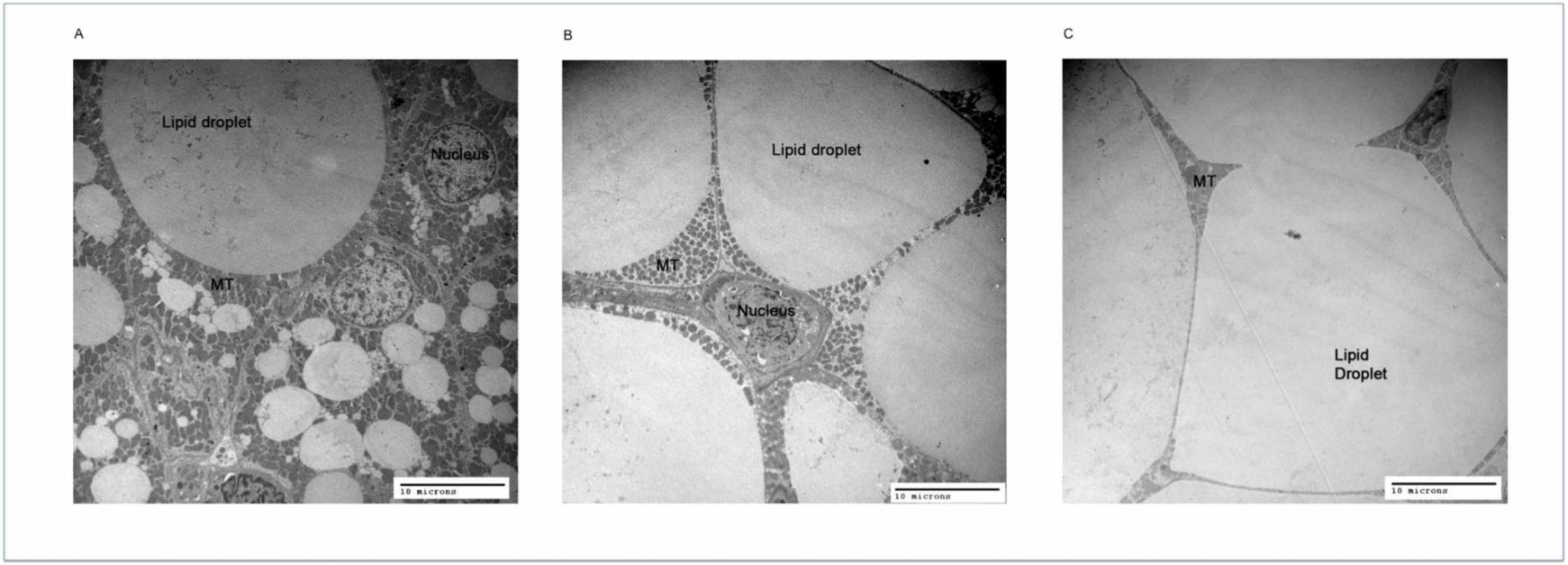

Figure 2.5: Transmission electron microscopy images (3000 × magnification) of brown adipose tissue. In neonates (A), early nursing (B), and late nursing (C) harp seal pups. The brown adipose tissue of neonates contains multilocular lipid droplets, and has a high mitochondrial density, but the brown adipose tissue of late nursing pups is characterized by few mitochondria and cells are filled with a single large lipid droplet. *MT* represents mitochondria.

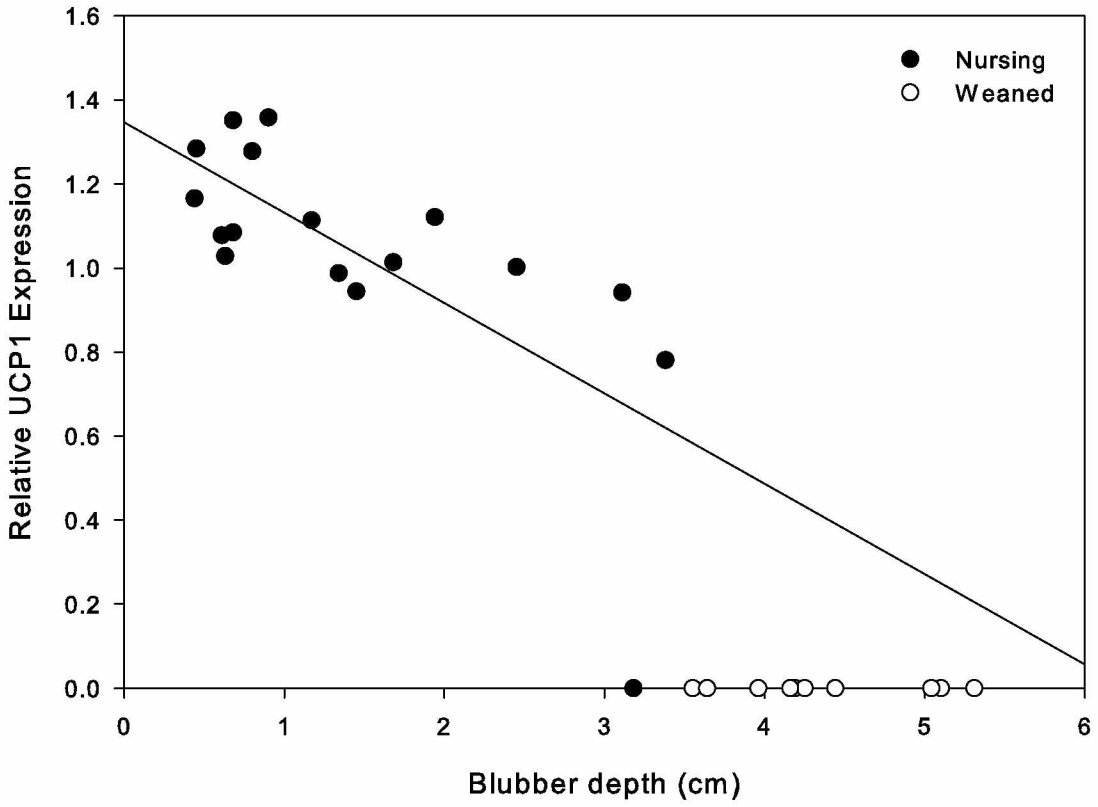

Figure 2.6: Regression of uncoupling protein 1 expression in relation to mean blubber depth. There is a significant negative linear correlation between the decline in uncoupling protein 1 expression and mean blubber thickness in nursing harp seals (black circles; $R^2 = 0.493$ P = 0.002). Weaned harp seals (open circles) did not express any UCP1, and were not included in the regression analysis.

Table 2.1: Mean morphometric and body condition values for harp seal. During nursing and early postweaning fast.

	Neonate	Early nursing	Late nursing	Early weaned	Late weaned
Sample size	10	3	4	5	5
Mass (kg)	9.8 ± 0.65^{a}	16.5 ± 0.29^{b}	28.6 ± 1.60^{c}	42.0 ± 2.37^{d}	29.8 ± 1.55^{c}
Standard length (cm)	82.8 ± 2.79^{a}	$87.3 \pm 3.48^{a,b}$	104.7 ± 2.73^{b}	101.2 ± 2.22^{a}	$89.6 \pm 5.50^{a,b}$
mass/length	0.10 ± 0.01^{a}	0.19 ± 0.01^{b}	0.28 ± 0.01^{c}	0.41 ± 0.02^{d}	0.34 ± 0.02^{c}
Total volume (L)	11.03 ± 0.89^{a}	15.72 ± 1.53^{a}	30.13 ± 3.50^{b}		
Surface area (m^2)	325.21 ± 13.89^{a}	388.25 ± 26.09^{a}	576.63 ± 41.42^{b}		
SA:V	29.63 ± 0.99^{a}	24.87 ± 1.03^{b}	20.73 ± 1.17^{c}		
Lean tissue volume (L)	9.56 ± 0.68^{a}	$11.16 \pm 1.04^{a,b}$	17.14 ± 2.24^{b}		
Blubber volume (L)	1.49 ± 0.17^{a}	4.55 ± 0.62^{b}	12.99 ± 1.47^{c}		
% Blubber by volume	13.75 ± 1.03^{a}	29.00 ± 2.08^{b}	43.50 ± 1.84^{c}		
Blubber thickness (cm)	0.80 ± 0.14^{a}	1.50 ± 0.10^{a}	2.00 ± 0.20^{b}	4.90 ± 0.21^{c}	3.90 ± 0.14^{d}
Fur thickness (cm)	2.081 ± 0.371^{a}	2.425 ± 0.163^{a}	2.598 ± 0.145^{a}	0.312 ± 0.030^{b}	0.192 ± 0.032^{b}

Superscripts indicate statistically significant differences ($P < 0.05$) between mean values for which there was effect of age. Measurements for volume calculations in early and late weaned animals were not obtained in this study.

Table 2.2: Mean conductivity values (± SEM). Sculp (fur + skin + blubber), blubber, and pelt (fur with skin) of harp seal pups during nursing and early postweaning fast and from harp seal adults.

	Neonate	**Early nursing**	**Late nursing**	**Early weaned**	**Late weaned**	**Adult**
Conductivity (*k*: W m^{-1} $°C^{-1}$)						
Sculp	0.14 ± 0.02[a]	0.17 ± 0.02[a,b]	0.28 ± 0.03[b]	0.18 ± 0.02[a,b]	0.16 ± 0.02[a,b]	0.19 ± 0.02[a,b]
Blubber	0.21 ± 0.05	0.22 ± 0.01	0.24 ± 0.02	0.19 ± 0.02	0.18 ± 0.02	0.19 ± 0.02
Pelt (fur with skin)	0.12 ± 0.02[a]	0.15 ± 0.02[a]	0.40 ± 0.08[b]	0.12 ± 0.01[a]	0.08 ± 0.01[a]	0.09 ± 0.01[a]

Superscripts indicate statistically significant differences ($P < 0.05$) between mean values for which there was effect of age on conductivity.

Table 2.3: Mean enzyme activity (± SEM; μmol min^{-1} g wet $weight^{-1}$). Cytochrome c oxidase (COX), citrate synthase (CS), and β-hydroxyacyl CoA dehydrogenase (HOAD) in the *Longissiumus dorsi* muscle and brown adipose tissue. Mean mitochondrial density (± SEM) of brown adipose tissue in harp seal pups was determined from images obtained by transmission electron microscopy that are represented in Figure 2.5.

	Neonate	Early nursing	Late nursing	Early weaned	Late weaned
Enzyme activity					
Muscle					
COX	6.25 ± 1.27^{a,b}	1.45 ± 0.43$^{a.b}$	1.05 ± 0.38^{a}	5.29 ± 0.78$^{a.b}$	7.61 ± 1.53^{b}
CS	49.68 ± 3.26^{a}	48.30 ± 2.22^{a}	47.38 ± 3.13^{a}	67.37 ± 0.80^{b}	75.08 ± 3.52^{b}
HOAD	70.61 ± 3.04	64.02 ± 15.65	83.39 ± 7.62	87.80 ± 3.01	98.42 ± 10.54
Brown adipose tissue					
COX	15.47 ± 2.17^{a}	3.70 ± 0.28^{a}	2.63 ± 0.30^{b}	2.15 ± 0.57^{b}	3.74 ± 0.74^{b}
CS	102.23 ± 15.75^{a}	94.15 ± 17.26^{a}	67.27 ± 10.20^{a,b}	13.51 ± 1.42^{b}	17.44 ± 1.65^{b}
HOAD	193.14 ± 31.44^{a}	114.57 ± 8.50^{a,b}	101.57 ± 21.34^{a,b}	17.09 ± 8.99^{b}	32.70 ± 8.79^{b}
Mitochondrial density (%)	13.88±2.00^{a}	4.50± 2.00^{b}	1.95± 0.87^{c}		

Data are reported as mean ± SEM. Superscripts indicate statistically significant differences ($P < 0.05$) between mean values for which there was effect of age.

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Chapter 3: Influence of mass and latitude on blubber fatty acid classes among the pinnipeds[1]

Abstract

The use of blubber as insulation is a trait unique to marine mammals, and its role in thermoregulation varies among pinnipeds. This makes it possible to test if proportions of FA in blubber vary in response to environment (temperature) and/or physiological (mass, insulation type, blubber thickness) constraints. We hypothesized that phocids, which create a temperature gradient across the blubber layer, may have higher proportions of PUFA in blubber to maintain flexibility at low temperatures when compared with otariids, which have an external temperature gradient through the fur layer, and maintain blubber near core body temperature. Further, phocid species inhabiting high latitude (colder) environments may have higher proportions of unsaturated FA than those in low latitude (warmer) environments to maintain fluidity of blubber lipids when exposed to low ambient temperatures. Among the phocids, there was a significant positive correlation between polyunsaturated FA (PUFA) and latitude ($p = 0.038$), which was mirrored by a significantly negative correlation between saturated FA (SFA) and latitude ($p < 0.001$). In contrast, otariids exhibited the opposite trends. We then compared the relative proportions of FA classes in blubber and muscle of three phocid species (harp, hooded, and Weddell seals) with similar thermal regimes to determine if the relative proportion of FA classes in blubber and muscle, and the difference between the two tissues, is similar among species. While there were species-specific differences in the FA classes in blubber, this was not the case for muscle. Our results indicate the FA composition of phocid blubber is influenced by ambient environmental conditions (represented through latitude), based on the need to modulate the thermal gradient through the blubber layer to maintain tissue flexibility. This is in contrast to skeletal muscle, where no thermal gradient exist, and the consistent temperature and underlying physiology influence the FA classes in this tissue.

[1] Pearson, L.E., Costa, D.P., Burns, J.M. Influence of mass and latitude on blubber fatty acid classes among the pinnipeds. Journal of Thermal Biology. In preparation.

Abbreviations

FA: fatty acid

FAME: Fatty acid methyl esters

LD: *Longissimus dorsi*

MUFA: monounsaturated fatty acid

PUFA: polyunsaturated fatty acid

SFA: saturated fatty acid

SA:V surface area to volume ratio

UFA: unsaturated fatty acid

WAT: white adipose tissue

3.1 Introduction

The amount of lipid stored in white adipose tissue (WAT) deposits is directly related to the overall condition and nutritional status of an individual (Pond 1978; Hanks 1981; Weber 2011; Champagne et al. 2012). Endogenous lipid stores contain much less water than carbohydrates and proteins (lipids ~ 10% water vs. carbohydrates ~ 30% water), which, in combination with their high energy density, accounts for their widespread use as caloric reserves (Pond 1998). Large lipid reserves have enabled animals to survive without an immediate food source, and inhabit areas previously unavailable because of adverse environmental conditions (Young 1976; Pond 1992; Fruhbeck et al. 2001). Unlike terrestrial mammals, most marine mammals do not store excess calories in WAT deposits distributed throughout the body (Pond and Ramsay 1992; Pond 1992). Instead, up to 75% of their total lipids are stored as triacylglycerols (TAG) in a hypodermal blubber layer (Reilly and Fedak 1990; Shero et al. 2014). This blubber layer acts as the primary heat loss barrier and aids in buoyancy control and body streamlining while diving (Webb et al. 1998; Sato et al. 2007; Aoki et al. 2011; Liwanag et al. 2012b).

The lipid profile of blubber (i.e., the fatty acids that make up the TAG stored in the blubber layer), generally reflects the fatty acids (FA) found in dietary sources (Budge et al. 2004; 2006; Iverson et al. 2004). However, there is modification (alteration of chain length and saturation) and differential allocation among tissues of ingested lipids that reflect structural and temperature adaptation and tissue specific needs. For example, the blubber of marine mammals

often has high levels of unsaturated FA (UFA), because UFA have lower melting points than saturated FA (SFA) (Irving et al. 1957). Thus, for individuals and species that maintain a thermal gradient across the blubber layer, this tissue may contain higher relative proportions of UFA, particularly polyunsaturated FA (PUFA), as a homeoviscous adaptation to maintain fluidity when the blubber layer is cool (Irving et al. 1957; Sokolov 1962; Wheatley et al. 2007; Trumble et al. 2010; Fowler et al. 2014). Under this scenario, species that live in colder environments and/or maintain a thermal gradient across internal blubber rather than external fur might have higher %UFA than those in warmer environments. In addition, because a thinner blubber layer provides less resistance to heat flux (Kvadsheim et al. 2002), individuals or species with thinner blubber insulation may also have higher %UFA. Larger animals have a lower surface area to volume (SA:V) ratio (Innes et al. 1990) and can carry a thicker blubber layer compared with smaller animals (Ryg et al. 1993). Thus, body size and condition (i.e., blubber thickness) might also influence the FA profile of blubber, with smaller animals or those with thinner blubber having higher %UFA in their blubber than larger animals.

Because the importance of the blubber layer in thermoregulation varies among pinniped species (Scholander et al. 1950; Gentry 1973; Irving 1973; Liwanag et al. 2012a; 2012b), it is possible to test the relative importance of latitude (proxy for ambient and sea surface temperature) and body mass or blubber depth on the relative proportion of FA classes in blubber. Adult phocids do not rely on fur for insulation, but instead maintain a thermal gradient across the blubber (Liwanag et al. 2012b). As a result, average ambient temperatures and the core-skin-air/water temperature gradient may influence the relative proportions of FA in blubber. Among the otariids, the extent to which blubber serves as a thermal barrier varies. In fur seals, a thick fur coat keeps skin and underlying tissues warm, and as such, fur seals may not need to have high levels of the more fluid UFA in their blubber layer as do phocids (Irving et al. 1957; Liwanag et al. 2012b). In contrast, sea lions rely on a combination of blubber and fur for thermoregulation (Gentry 1973; Liwanag et al. 2012b), and there is a thermal gradient in the blubber layer, suggesting that UFA content may need to be higher than in fur seal species from similar environments. Overall, phocid species that inhabit high latitude (colder environments) may have more PUFA in their blubber than species that inhabit low latitudes (warmer environments) due to the varying fluidity and flexibility of FA classes at different temperatures. Further, species that

do not rely on blubber for thermoregulation may not show any correlation between FA class composition with latitude, and may have fewer PUFA in their blubber.

In addition to their blubber layer, marine mammals store large concentrations of lipid in their skeletal muscles, presumably to fuel oxidative metabolism while diving (Davis et al. 1991; Kanatous et al. 2002; Trumble et al. 2010). This is particularly true for phocids, which are known to rely heavily on endogenous lipids and lipids transported from WAT to skeletal muscle to fuel oxidative metabolism (Davis and Kanatous 1999; Kanatous et al. 2002), and TAG can account for ~15% of skeletal muscle volume (Trumble et al. 2010). Because muscle temperature is kept close to core body temperature (Ponganis et al. 1993; Noren et al. 2008), the relative proportions of SFA may be higher in muscle than blubber. In addition, because core/muscle temperature is similar among all marine mammal species, the FA class proportions in muscle may be similar among species, given that phocids also share a similar underlying lipid based metabolism (Kanatous et al. 1999).

We compare the relative proportions of FA classes in blubber of phocid species from literature values to determine if latitude (as a proxy for air and sea surface temperature) and body mass influence blubber FA among phocids. We hypothesize that blubber of species that haul out in cold environments (i.e., high latitude) will have a higher proportion of UFA in their blubber than species in low latitude environments. Additionally, we test whether there are differences in the relative proportions of FA classes in blubber between phocids and otariids, which may correlate with their reliance on blubber for insulation. We hypothesize that the difference in the role of blubber in thermoregulation results in phocids having greater relative proportions of UFA than otariids. We further investigate if within each family, higher latitude species have greater relative proportions of UFA in the blubber than lower latitude species, and if larger species store a greater relative proportion of SFA than smaller species, as would be expected based on changes in thermodynamics associated with increased body size.

We then compare the FA classes in blubber and muscle among three phocid species: harp (*Pagophilus groenlandicus*), hooded (*Cystophora cristata*), and Weddell (*Leptonychotes weddellii*). These three species have similar thermal regimes and life history, allowing us to determine if FA class composition of blubber and muscle, and the difference between the two tissues, is similar among species. We hypothesize that the relative proportions of FA classes in blubber will be different among species, as mass differences effect thermoregulation, though

muscle FA classes may be similar among species as energy metabolism may result in similar allocation of FA. Finally, we determine if species with larger body mass or thicker blubber have a greater proportion of SFA and fewer PUFA in the blubber or muscle.

3.2 Methods

3.2.1 Animal captures and sample collection

Adult female harp (n = 5) and hooded seals (n = 5) were captured in 2008 in the Gulf of St. Lawrence, Canada (47.60, -62.22), and in 2011, an additional 4 hooded seals were captured in the "West Ice" off Greenland (72.40, -14.25). Adult female Weddell seals (n = 8) were captured in McMurdo Sound, Antarctica (~ -77, 165) in October and November (austral spring), 2010 and 2011. Harp and hooded seals were sacrificed using methods approved for scientific harvest in Canada or Norway. Within 30 minutes *post-mortem*, subcutaneous blubber thickness at the mid-dorsal site was measured using a ruler, and *Longissimus dorsi* (LD) and full thickness blubber samples were collected. Weddell seals were anesthetized with an intramuscular injection of 1.0 mg kg^{-1} tiletamine/zolazepam HCl, followed by intravenous injections of ketamine/diazepam (1:1 ratio, 100 mg ml^{-1} and 5 mg ml^{-1}) as necessary to maintain sedation (0.20 mg kg^{-1} ketamine, ~0.01 mg kg^{-1} diazepam). Subcutaneous blubber thickness at the mid-dorsal site (Shero et al. 2014) was measured using a SonoSite Vet180Plus portable ultrasound with a 3.5 MHz convex transducer (SonoSite Inc., Bothell, WA, USA). Blubber (full thickness) and muscle (LD) biopsies were collected separately using a 6 mm biopsy punch (blubber) and cannula (muscle). All tissue samples were frozen in liquid nitrogen, and stored at -80 °C until analysis. Total body mass (± 1 kg) was determined by direct weighing using a spring scale (harp and hooded seals) or a load-cell scale (Weddell seals; MSI-7200-IT Dyna-Link digital dynamometer, Measurement Systems International, Seattle, WA, USA). All seals were sampled while lactating, and all samples were collected within the first quartile of lactation (harp seals: 1–2 days post-parturition; hooded seals: < 1 day post-parturition; Weddell seals: 1-5 days post-parturition).

3.2.2 Fatty acid analysis

Samples of blubber (0.200–0.500 g) and muscle (0.030–0.120 g) were thawed and weighed to the nearest 0.001 g (wet mass). Lipid was extracted in chloroform:methanol 2:1 (Folch et al. 1957) on a Soxhlet apparatus. Fatty acid methyl esters (FAME) were prepared from

the lipid extract using an acid catalyzed esterification as described by Budge et al. (2006). FAME were extracted into dichloromethane (DCM: HPLC grade), concentrated, and brought up to volume (10 mg FAME ml DCM^{-1}). FAME of blubber and muscle samples from harp, hooded and Weddell seals were analyzed on a Varian 3900 GC-FID (Varian Inc., Walnut Creek, CA, USA) at Baylor University following protocols of Budge et al. (2006) with the following modifications: Column CP-Select for FAME (CP419) 100 m × 0.25 mm ID × 0.25 μm. The injector temperature was 250 °C with a 1 μl injector split ratio of 50:1. Column flow was 1.0 ml min^{-1} programmed at 210 °C for 9.0 min and ramped at 15 °C min^{-1} to 260 °C for 7.7 min. Detector temperature was set at 300 °C with a hydrogen flow of 30 ml min^{-1} and airflow of 300 ml min^{-1}. FAME peaks were identified based on retention times of a known standard mixture (Supelco 37 Component FAME Mix; Sigma-Aldrich Co., St. Lewis, MO, USA). Individual FA concentrations in the sample were determined from a 5-point standard curve created using the Supelco FAME Mix. The standard curve included a range of concentrations representative of FA in the samples analyzed. FA identities were verified via mass spectroscopy (Hewlett Packard 6890 GC and 5973 Mass selective detector; Hewlett Packard, Palo Alto, CA, USA) in four samples (two blubber, two muscle). Peak identification and integration of the 37 FA included in the standard were manually checked and corrected if necessary for each sample. FAME were described by shorthand nomenclature: [carbon number]:[number of double bonds]*n*[position of first double bond from the methyl end] according to the IUPAC (IUPAC-IUB Commission on Biochemical Nomenclature 1976). All FA data are presented as percent contribution of the particular FA to the sum total of all FA detected in sample (mean ± 1 SD). Relative proportions of FA by class were determined by summing the percent of all FA within each class ($\%\Sigma FA_C$: relative proportion of saturated [%ΣSFA], monounsaturated [%ΣMUFA] or polyunsaturated [%ΣPUFA] FA).

3.2.3 Data analysis

Patterns among pinnipeds

To determine if blubber FA class proportions differed among species that inhabit different latitudes and/or that rely more or less on blubber as their primary thermal barrier, we collated FA class data from the literature from 12 phocid and seven otariid species (Table 3.1). When multiple FA values were available for a given species all values were retained, and mean

and error terms were reported. Within species replication originates from different geographic populations, sampling locations, collection years, age/sex components, or analytical labs. The body mass values used in our analyses are reported in Table 3.1. When available, body mass was taken from the same study as the FA data, or other studies of the same population, or average body mass from Krüger et al. (2014). Sample collection latitude (Table 3.1) was obtained from the same studies as the FA data.

Principle Component Analysis (PCA) was used to examine differences between phocids and otariids based on the relative proportions of FA classes in blubber. Because there was clear separation of the two pinniped families, two separate PCA and PCA biplots were run to examine the within family correlations among mass, latitude, and FA class of phocids and otariids. Linear mixed models were fit using maximum likelihood to determine the direction and magnitude of the correlations between latitude and mass on the $\%\Sigma FA_C$ in blubber among species of phocids. Mixed models were not run on the data from otariids, because four of the ten studies that reported blubber FA data contained data from fewer than three individuals (Table 3.1). Random factors in the phocid model included species and age/sex class (adult female or combined ages and sexes). Models were weighted using the inverse estimated variance method (*n*/variance) to account for the unequal variance and sample size underlying the population mean. If covariates were not statistically significant ($p > 0.05$), we assumed there was minimal evidence of a correlation, and these terms were removed from the model. Mass data were log-transformed before analysis to approximate normality. All analyses were completed using the software R (v 3.1.2, R Development Core Team, www.R-project.org), and linear mixed effect models were fit using the nlme package in R (Pinheiro et al. 2009). Significance was considered at $p < 0.05$, and data are reported as mean ± 1 SD.

Differences between blubber and muscle

We used MANOVA with Bonferroni correction to test if the relative proportion of FA classes (%ΣSFA, %ΣMUFA, or %ΣPUFA) in blubber and muscle differed among species/populations of harp, hooded, and Weddell seals. Paired *t*-tests were used to determine if there were significant differences between tissues within each species. Finally, we calculated the difference in each FA class between blubber and muscle samples (ΔFA_{B-M}) for each species, and used a MANOVA with Bonferroni correction to determine if ΔFA_{B-M} differed among species/population. Linear mixed effects models were used to examine correlations between

body mass, blubber thickness, the interaction between mass and blubber thickness, and the relative proportion of FA classes in blubber and muscle. Species was included as a random factor. If covariates were not statistically significant ($p > 0.05$), we assumed that there was minimal evidence of a correlation, and these terms were removed from the model. Mass data were log transformed and FA data were arcsine square root transformed prior to analysis to approximate normality.

3.3 Results

3.3.1 Patterns among pinnipeds

The FA class composition of the 12 species of phocids differed from the composition of the seven otariid species for which data were available. In the phocids, %ΣMUFA was the dominant FA class of blubber (Figure 3.1A), whereas in the otariids, no single FA class was dominant (Figure 3.1B). This difference was reflected in the PCA of the combined phocid and otariid data (Figure 3.2), which showed separation of the two families based on blubber FA composition. PC1 accounted for 38.7% of the variation and represented a gradient from lower to higher %ΣMUFA, whereas PC2 accounted for 34.4% of the variation and represented a gradient from high %ΣSFA and low %ΣPUFA to low %ΣSFA and high %ΣPUFA. On average, otariids had a higher %ΣSFA (23.43 ± 4.28% vs 13.99 ± 3.37%), a lower %ΣMUFA (46.95 ± 8.95% vs 55.08 ± 8.35%), but similar levels of %ΣPUFA (29.50 ± 11.55% vs 29.13 ± 9.87%) in their blubber when compared with phocids.

The within-family PCA (Figure 3.3) highlights the differing role of blubber between phocids and otariids, as the FA class structure differed by family as seen in the opposite vector pattern representing latitude, %ΣPUFA, and %ΣMUFA. Among the otariids (Figure 3.3A, the first two PC accounted for 83.1% of the total variance in the blubber FA classes. PC1, which accounted for 58.0% of the variance, represented a gradient from animals with higher %ΣMUFA, living at higher latitude, to animals with high %ΣPUFA at lower latitudes. PC2, which accounted for 25.1% of the variance, represented a gradient in %ΣSFA. Interestingly, neither PC1 nor PC2 separated species based on whether their primary insulation was blubber (three species of sea lion) or fur (three species of fur seal).

Among phocids, the first three PC accounted for 88.0% of the total variance in the proportion of FA in each class. PC1 and PC3 separated species based on latitude. PC1 accounted for 46.2% of the variation, and the biplot vectors along this PC showed the correlation between latitude and %ΣPUFA (Figure 3.3B). PC1 also revealed the gradient among species with high %ΣMUFA and those with high %ΣPUFA, though %ΣMUFA was not predicted by body mass or latitude in the mixed models. PC2 (25.9% of the variance) represented a gradient from species with low %ΣMUFA to those with higher %ΣSFA and high female body mass. Additionally, PC2 split the phocid species by subfamily (Monachinae vs. Phocinae), with some of the phylogenetic divergence by tribe (Miroungini vs. Lobodontini, and Phocini vs. bearded (*Erignathus barbatus*) and gray (*Halichoerus grypus*) seals) further captured by PC1.

The mixed models of the phocid species data provided similar results as the PCA analysis. While mass was not a significant predictor of the proportion of any fatty acid class, there was a significant positive correlation between blubber %ΣPUFA and latitude ($p = 0.038$), and a significant negative correlation between blubber %ΣSFA and latitude ($p < 0.001$; Table 3.2). The slopes of these correlations, while opposite in direction, were similar in magnitude. Among the seven species for which data were available for populations across a latitudinal gradient (Table 3.1), all but ringed seals (*Pusa hispida*) showed a positive correlation between %ΣPUFA of blubber and latitude, although none of these within-species correlations were significant. The relative proportion of %ΣMUFA in blubber was not predicted by latitude or mass.

3.3.2 Differences between blubber and muscle

We found the relative proportions of FA classes in both blubber and muscle were broadly similar among harp, hooded, and Weddell seal adult females (Table 3.3). Blubber had higher %Σ MUFA and lower %ΣSFA in all but Weddell seals (Table 3.3). Differences in FA class proportion of blubber occurred both within and between species, suggesting that differences were due to habitat rather than phylogeny. In contrast, muscle was not dominated by a single FA class; instead ~80% of FA were split fairly evenly between %ΣSFA and %ΣMUFA, with %ΣPUFA contributing the remaining 20%. There were no significant differences among species in the relative proportion of any particular FA class in muscle. When comparing blubber and muscle proportions of fatty acid classes within individuals across species, there were broadly similar patterns (Figure 4). In all species/populations, muscle contained significantly greater %ΣSFA ($p = 0.001$ for all), and significantly lower %ΣMUFA than blubber (harp: $p = 0.049$,

hooded$_{Canada}$: $p = 0.001$; hooded$_{Greenland}$: $p = 0.011$; Weddell: $p = 0.005$). However, both harp seals and Greenland hooded seals had significantly greater %ΣPUFA in their blubber ($p = 0.025$ and $p = 0.008$, respectively; Figure 3.4) than their muscle. Neither Canadian hooded seals nor Weddell seals exhibited a significant difference in %ΣPUFA of muscle and blubber.

Mixed models showed blubber thickness was a significant predictor of %ΣSFA in blubber ($p = 0.039$; Table 3.2), with a 1 cm increase in blubber thickness leading to a decrease in the relative abundance of %ΣSFA by 2 percentage points. Similarly, a 1% increase in body mass was associated with a ~0.1 percentage point increase in %ΣSFA in blubber, but a decrease in %ΣPUFA by ~0.01 percentage points. The interaction term between body mass and blubber thickness was not significant for any FA class, and neither blubber depth nor mass were significant predictors of the relative proportion of %ΣMUFA in blubber.

In muscle, heavier seals had significantly greater %ΣSFA and %ΣMUFA (%ΣSFA: $p = 0.011$; %ΣMUFA: $p = 0.039$), but a significant lower relative proportion of %ΣPUFA ($p = 0.039$). A 1% increase in body mass was associated with a decrease in %ΣPUFA by 0.5 percentage points. Seals with thicker blubber had a greater %ΣMUFA ($p = 0.016$), but less %ΣSFA ($p = 0.038$). Blubber thickness was not a significant predictor of %ΣPUFA in muscle. Both the effects of mass and blubber thickness on %ΣMUFA and %ΣSFA were further compounded by a significant interaction term between mass and blubber thickness resulting in a positive correlation with %ΣSFA ($p = 0.015$), but negative correlation with %ΣMUFA ($p = 0.042$). Neither blubber thickness nor the interaction between mass and blubber thickness were significant predictors of %ΣPUFA. In general, among harp, hooded, and Weddell seals, animals with thicker blubber had less %ΣSFA in blubber and muscle, and greater %ΣMUFA in muscle. Heavier animals had greater %ΣSFA in their blubber and muscle, greater %ΣMUFA in their muscle, and less %ΣPUFA in their blubber and muscle. Additionally for %ΣSFA, the magnitudes of these correlations were more pronounced in muscle than in blubber (Table 3.2).

3.4 Discussion

Overall, this study reveals that the reliance by phocids on a thick blubber layer for insulation is associated with a FA class profile that has higher relative proportions of UFA compared with the blubber of otariids. This difference may be a result of interactions between ambient and tissue temperatures in blubber; this idea is also supported by the significant increase

in %ΣPUFA in blubber across phocid species as latitude increases. Furthermore, heavier phocid species tended to have relatively more %ΣSFA in blubber, likely due to the lower thermal gradient within the tissue as compared with smaller phocid species. The absence of thermal constraints on FA classes in muscle is apparent; skeletal muscle has greater %ΣSFA and less %ΣMUFA than blubber, and the effect of individual body mass and blubber thickness on muscle lipids differed from that in blubber.

The relative proportions of FA classes in blubber differed between phocids and otariids, as did the effect of latitude and body mass. In contrast to our predictions based FA flexibility with temperature, otariids living at high latitudes had lower %ΣPUFA and higher %ΣSFA in their blubber than those living at low latitudes. This pattern is likely a reflection that most otariids maintain a temperature gradient between the body core and ambient environment across the fur layer (Bartholomew and Wilke 1956; Gentry 1973). As the conductivity of fur is very low, otariids maintain their skin temperature, and thus, the hypodermal blubber layer, near core body temperature (Bartholomew and Wilke 1956; Gentry 1973; Willis et al. 2005; Liwanag et al. 2012a; Liwanag et al. 2012b). Because the blubber layer experiences little temperature flux in otariids, there may be no need to regulate %ΣPUFA or %ΣSFA to maintain blubber flexibility. Though sea lions rely on a combination of fur and blubber for thermoregulation, we did not see any differences in the proportions of blubber FA classes between sea lions and fur seals. This may be a result of the small number of otariid species included in this study. Nevertheless, whereas sea lion skin temperatures are likely lower than those of fur seals (Nienaber et al. 2010), the skin temperature of sea lions may still be near enough to core temperature that the modulation of the relative proportions of FA in the blubber layer is unwarranted (Willis et al. 2005; Nienaber et al. 2010; Liwanag et al. 2012a). The lack of correlation between FA class and latitude in otariid blubber as seen in this study is similar to that observed in fur-reliant semiaquatic and terrestrial mammals (Käkelä and Hyvärinen 1996). Käkelä and Hyvärinen (1996) showed superficial and deep fat deposits of Canadian beaver (*Castor canadensis*), Eurasian otter (*Lutra lutra*), and raccoon dog (*Nyctereutes procyonoides*), all of which had uniform FA composition regardless of proximity to the skin. In contrast, FA in blubber adjacent to the skin of ringed seals (*Pusa hispida*) were more unsaturated than those of deep blubber, where temperatures were warmer and more consistent (Käkelä and Hyvärinen 1996). We did not have sufficient sample size to analyze the magnitude of latitude and body mass effects on the

relative proportion of FA classes in the blubber of otariids. However, the otariid species included in this study range across 32° latitude, and include species from each of the proposed branch points in otariid phylogeny (Higdon et al. 2007; Berta and Churchill 2012). Thus, the trends seen among them are likely representative of the whole lineage.

Phocid species at high latitudes had greater relative proportions of %ΣPUFA and lower proportions of %ΣSFA in their blubber than species living at low latitudes. This trend is also apparent within species; among the species for which there were data of multiple populations, those populations living at higher latitudes typically had higher %ΣPUFA than those living at lower latitudes (Table 1). This highlights both among and within-species plasticity of the blubber FA composition and may reflect both adaptation and acclimation to meet different environmental conditions. The correlations between FA class and latitude indicate that species living at higher latitudes may be adapted to store greater %ΣPUFA and less %ΣSFA in blubber to maintain FA fluidity, and thus, blubber flexibility (Irving et al. 1957; Sokolov 1962; Käkelä and Hyvärinen 1996), creating what appears to be a tradeoff between the relative proportions of %ΣPUFA and %ΣSFA. Alternatively, this internal modulation of blubber lipid composition may be partially a result of high latitude prey species having high %ΣPUFA (Sidell 1991; Mayzaud et al. 2011; Jo et al. 2013). For phocids living at high latitudes, this would result in diets naturally high in PUFA, which could be preferentially allocated to blubber. While there is variation in the FA composition of prey species by region and year (Daslgaard 2003), none of the prey species of harp, hooded, and Weddell seals (Table 3.4) are exceptionally high in %ΣPUFA (Hooker et al. 1999; Dahl et al. 2000; Dahl et al. 2003; Jo et al. 2013). Zooplankton at high latitudes are enriched in wax esters, which are digested and deposited as long-chain MUFA in zooplanktonivorous fish (Dalsgaard et al. 2003; Tocher 2003); this result is especially apparent in the primary prey of hooded seals, Arctic cod (*Boreogadus saida*), which are especially high in %ΣMUFA (Table 3.4). The underlying cause of the pattern observed in phocids is difficult to determine without direct analysis of dietary FA from the region and year of phocid species presented here.

Overall, heavier phocid species had more %ΣSFA and less %ΣPUFA in their blubber than lighter species. This might be expected as larger species have thicker blubber, thus greater thermal resistance. Given similar ambient temperatures, seals with thicker blubber are able to maintain higher temperatures through a larger proportion of blubber (Hart et al. 1959). Warmer

temperatures within the blubber would naturally maintain flexibility and animals would not need to modulate the FA class composition to counteract low temperatures (Irving et al. 1957; Käkelä and Hyvärinen 1996). The same temperature gradient across thinner blubber would be steeper, and SFA may become less fluid (Irving and Hart 1957; Hart et al. 1959; Ryg et al. 1993). When exposing a greater thickness of blubber to cold temperatures, more PUFA would be incorporated in blubber to maintain flexibility (Irving and Hart 1957; Irving et al. 1957). In harbor seals (*Phoca vitulina*), temperature variation was reflected in blubber down to 1.5 cm, and the underlying blubber was euthermic (Hart and Irving 1959; Irving and Hart 1957). Further, mass and blubber thickness are the primary influences on both convective and conductive heat loss, superseding ambient conditions (Mellish et al. 2014). Our results showed body mass and blubber thickness were also significant predictors of the relative proportions of FA classes among harp, hooded, and Weddell seals. However, within these species, as body mass or blubber thickness decreased, %ΣSFA in blubber increased. This effect was tempered by the interaction term between body mass and blubber thickness. Smaller individuals or those with thinner blubber may not be able to maintain a skin temperature near ambient as well as larger animals (Hart et al. 1959), resulting in blubber temperatures nearer to core temperature, and thus greater allocation of SFA to this tissue (Hart et al. 1959; Noren et al. 2008; Erdsack et al. 2012; Mellish et al. 2013; 2014). Despite this, the species with the thinnest blubber layer and lightest mass we measured, the harp seal, had the greatest %ΣPUFA in the blubber layer, while the largest species, the Weddell seal, had the greatest %ΣSFA. These correlations with body mass and blubber thickness were also observed between the two populations of hooded seal; the smaller and thinner individuals in Greenland had higher %ΣPUFA than the larger individuals from Canada. This overall pattern among these three species fit within the phocid-wide pattern we observed.

Whereas our phocid-wide PCA biplot showed a positive correlation between body mass and %ΣSFA, this correlation was not significant in the mixed models. As body mass influences FA classes in blubber at an individual level (see our hooded seal data), it is not surprising that the average body mass for a species or population was insufficient to describe any variation in the relative proportions of FA classes. It is interesting to note that the largest species, those that belong to the subfamily Monachinae: southern elephant seal (*Mirounga leonina*), northern elephant seal (*Mirounga angustirostris*), and Weddell seal, consistently had more %ΣSFA and less %ΣPUFA in their blubber than might be expected based on latitude (Figure 3.3A).

Compared with smaller phocids, Weddell seals and northern elephant seals have low tissue-level aerobic capacities (Reed et al. 1994; Kanatous et al. 2002; Watson et al. 2007; Kanatous et al. 2008; Moore et al. 2014), thus less metabolic heat production and FA turnover. Castellini et al. (2009) showed that Weddell seals have thicker blubber, and thus, more insulation for their size than other species at similar latitudes. These larger species may compensate for low metabolic heat production by having thicker insulation than expected based on body mass and thermodynamics alone (Castellini et al. 2009), and thus higher proportions of %ΣSFA in the blubber.

While thermoregulatory considerations have an important influence on the FA composition of blubber, the impact of diet on this pattern cannot be discounted (Bradshaw et al., 2003; Dalsgaard et al., 2003; Iverson et al., 2004; Iverson et al., 1997). For example, the blubber of crabeater seals (*Lobodon carcinophagus*), which feed almost exclusively on PUFA rich Antarctic krill (*Euphasia superba*) (Hückstädt et al. 2012; Forcada et al. 2012), contains the highest %ΣPUFA of all phocids included in this study (Table 3.1). For species with more diverse diets, such as harp, hooded, and Weddell seals (Burns et al. 1998; Tucker et al. 2009), there is greater variation in the FA class composition of prey species (Table 3.4), and the FA classes in the blubber represent a mixture of these sources.

Our results showing the correlations between latitude and the relative contribution of blubber FA classes in phocids are similar to those observed in cetaceans. Smaller cetacean species and those living at higher latitudes have greater dietary and biosynthesized %ΣPUFA and biosynthesized wax esters in their blubber layer to maintain fluidity in a cold environment (Worthy and Edwards 1990; Samuel and Worthy 2004; Dunkin et al. 2005). Among cetaceans, there is also evidence that the relative %UFA of blubber increases in the cold winter months, or when animals migrate to colder water habitats (Lockyer et al. 1984; Worthy and Edwards 1990; Samuel and Worthy 2004; Dunkin et al. 2005). Cetacean blubber is highly structured, and FA are stratified into biochemically and histologically distinct layers (Krahn et al. 2004; Strandberg et al. 2008; 2011). This stratification allows FA in the inner layers to be quickly mobilized for transport (Lockyer et al. 1984; Lockyer 1987; Koopman 2007; Strandberg et al. 2008). Phocid blubber has three layers; an inner layer closest to the core that is readily mobilized, a middle layer, and a superficial layer closest to the skin (Strandberg et al. 2008; 2011). While we compared the relative proportions of FA classes in full thickness blubber samples, the impact of

both the latitude and body mass may differ by layer (Strandberg et al. 2011). The superficial layer of blubber has selective incorporation and mobilization of UFA, and higher *in situ* modification of FA by Δ9-desaturase than both other layers (Irving and Hart 1957; Hart and Irving 1959; Strandberg at al. 2011). Adjusting lipid viscosity in this layer is likely key as this is where the temperature gradient between core and ambient is steepest (Irving and Hart 1957; Hart and Irving 1959; Strandberg et al. 2008; 2011). Considering the FA of only the superficial layer of blubber may reveal stronger correlations between latitude and %ΣSFA or %ΣPUFA among phocids (Strandberg et al. 2011).

The relative proportion of FA classes in muscle did not differ among phocid species, though body mass and blubber thickness were important predictors of FA class proportions in across muscle. There were significant differences in the relative proportion of each FA class in blubber, different diets of each species, and different FA class proportions among prey species (Table 3.4; Burns et al. 1998; Tucker et al. 2009). As such, the similarity in the FA class proportions of muscle suggests that the warmer, consistent temperature regime of skeletal muscle may allow storage of SFA in greater relative proportions than in blubber. Saturated FA are the most energy dense FA for a given chain length (Gurr et al. 2002), thus providing the added benefit of dense energy stored in skeletal muscle. Though all phocids rely primarily on lipid metabolism (Davis et al. 1991; Kanatous et al. 1999; Kanatous et al. 2002; Davis and Williams 2012), there are differences in oxidative enzyme concentrations (Kanatous et al. 2008; Burns et al. 2007), myoglobin concentrations (Kanatous et al. 2002; Burns et al. 2007), and diving behavior (Williams et al. 2000) among species. These within-family differences may lead to differential use of individual FA in muscle (Price et al. 2011). The influence of body mass and blubber thickness on the relative proportions of FA classes in muscle among species is likely related to the effect that both mass and body condition have on metabolic rate (Kleiber 1975; Aarseth et al. 1999; Nagy 2005; Weibel and Hoppeler 2005).

In summary, among phocids there are clear correlations between latitude and the relative proportions of FA classes in blubber. This pattern is evident despite large variation in the FA composition of the diet, body mass, and blubber thickness among species. This suggests that phocids are able to modulate FA in blubber to deal with different environmental conditions. However, more research is needed to determine if this is a result of an adaptation of the seal, or simply a result of diet and prey species naturally having greater %ΣPUFA in high latitude

environments. There are clear differences in the FA composition of blubber and muscle, and mass and body condition appear to contrarily influence the FA in these tissues. In depth consideration of lipid turnover and biosynthesis rates in each tissue may further elucidate these correlations. Our findings indicate that blubber is not merely a static storage site, but instead a highly dynamic tissue, the composition of which reflects environment, thermodynamics, diet, and body mass. In contrast, variation in muscle lipid stores is attributable to body mass and blubber thickness rather than latitude. While we did not have enough muscle or blubber samples for any individual species to test for differences in phocid individual FA acids, it may be relatively few individual FA within a class that contribute to a tradeoff between flexibility and thermoregulation for an individual. Further studies are needed to determine if blubber is influenced uniformly by temperature, or if the most superficial layer is influenced disproportionately by temperature, and if these same environmental and physiological factors influence the individual FA that vary with each tissue.

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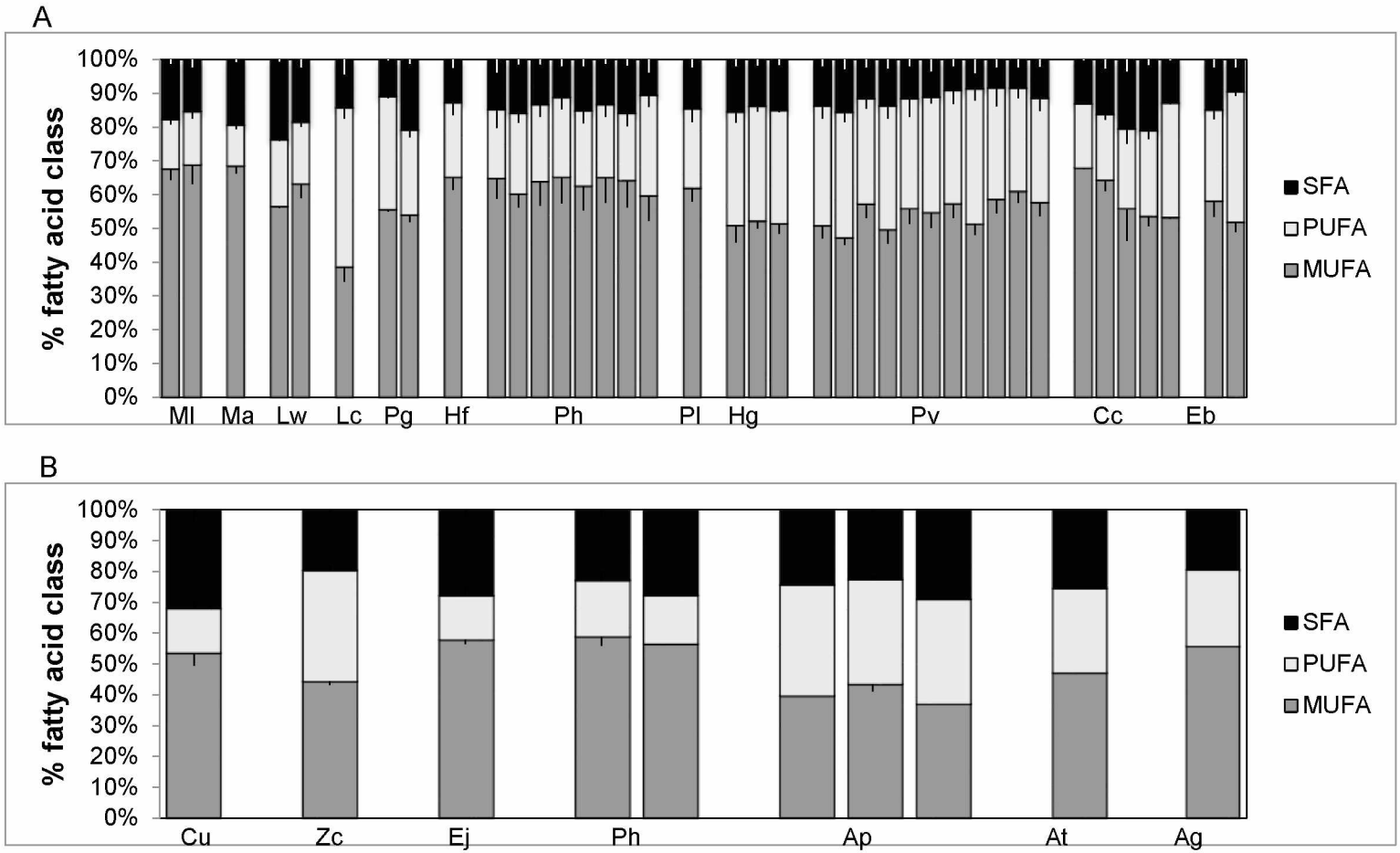

Figure 3.1: Fatty acid class composition (mean ± 1 SD) of blubber. (A) 12 species of phocid (true seals) and (B) 7 species of otariids (sea lions and fur seals). Within species order by populations living at increasing latitude. (A) Ma = *Mirounga angustirostris*, Ml = *Mirounga leonina*, Lw = *Leptonychotes weddellii*, Lc = *Lobodon carcinophaga*, Pg = *Pagophilus groenlandicus*, Hf = *Histriophoca fasciata*, Pv = *Phoca vitulina*, Pl = *Phoca largha*, Hg = *Halichoerus grypus*, Ph = *Pusa hispida*, Cc = *Cystophora cristata*, Eb = *Erignathus barbatus*. (B) Sea lions: Ej = *Eumatopius jubatus*, Ph = *Phocarctos hookeri*, Zc = *Zalophus californianus*; Fur seals: Ap = *Arctocephalus pusillus pusillus*, Ag = *Arctocephalus gazella,* Cu = *Callorhinus ursinus.*

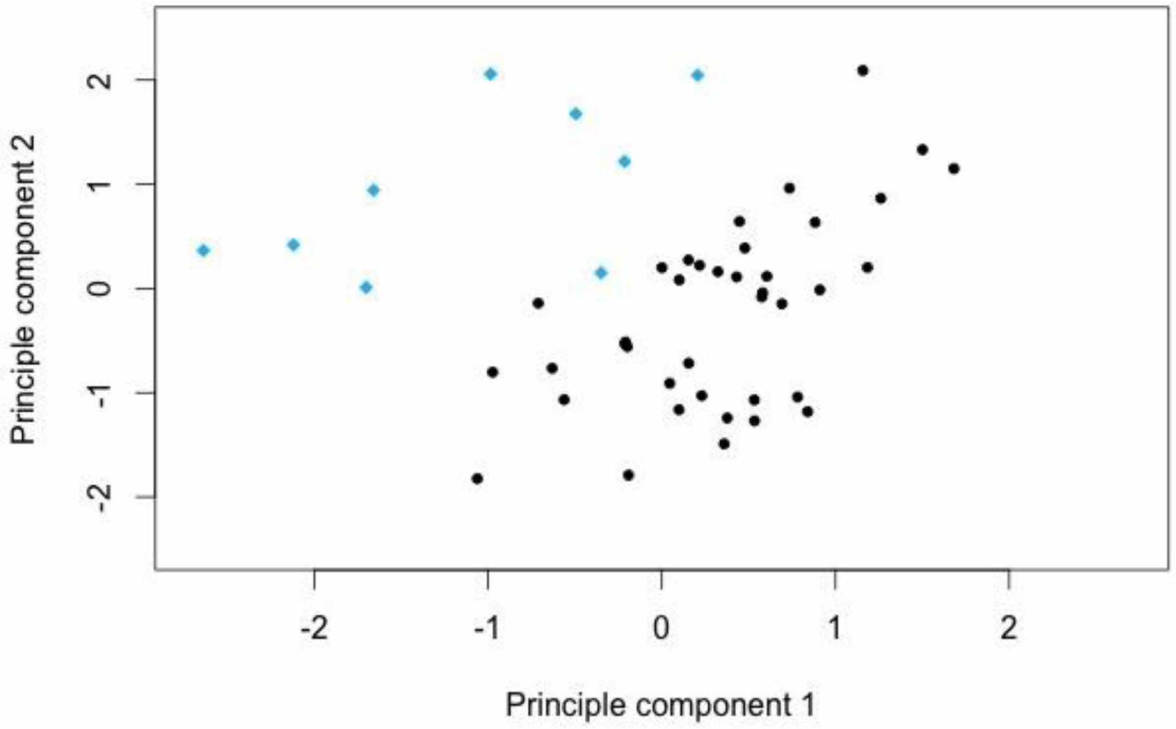

Figure 3.2: Plot of principle components 1 and 2. Principle component analysis of the relative proportion of fatty acid class composition of blubber in phocids (black circle) and otariids (blue diamond).

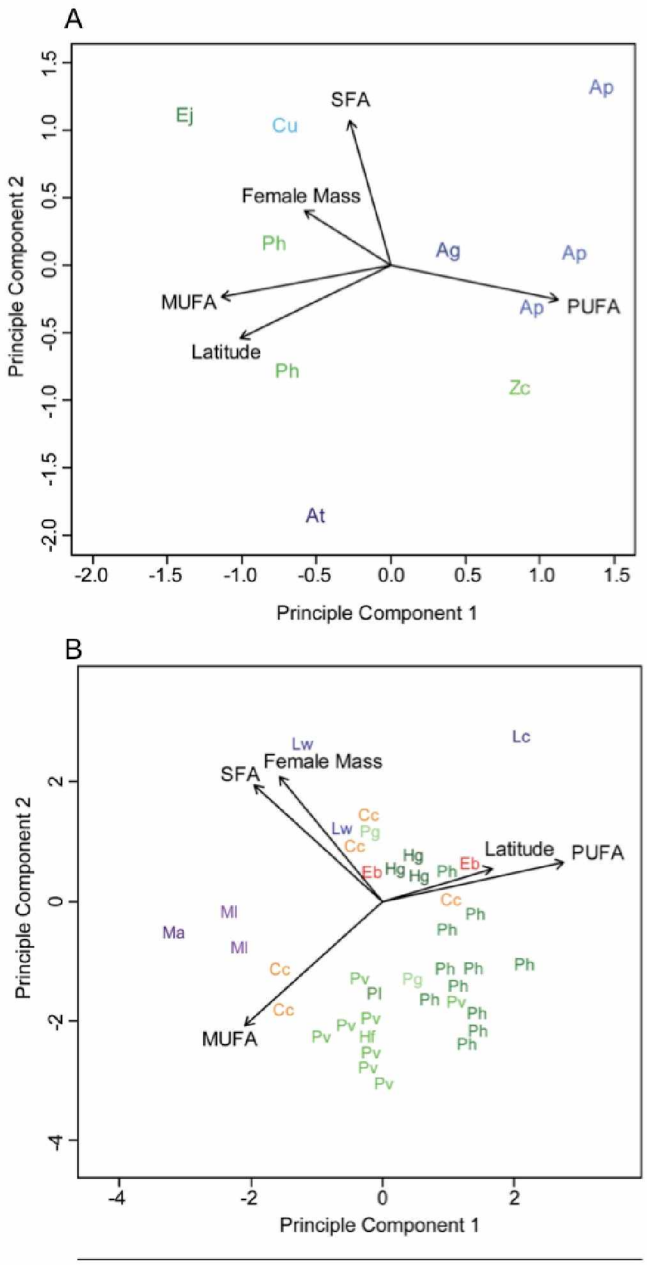

Figure 3.3: Principle component analysis biplot of principle components 1 and 2. Panels show correlations among latitude and mass and the relative proportions of fatty acid classes (SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid) in blubber of multiple otariid (A) and phocid (B) species and populations. Vectors represent the variables included in the analysis, and move in the direction of increasing values for that variable. (A) Ma = *Mirounga angustirostris*, Ml = *Mirounga leonina*, Lw = *Leptonychotes weddellii*, Lc = *Lobodon carcinophaga*, Pg = *Pagophilus groenlandicus*, Hf = *Histriophoca fasciata*, Pv = *Phoca vitulina*, Pl = *Phoca largha*, Hg = *Halichoerus grypus*, Ph = *Pusa hispida*, Cc = *Cystophora cristata*, Eb = *Erignathus barbatus*. (B) Sea lions: Ej = *Eumatopius jubatus*, Ph = *Phocarctos hookeri*, Zc = *Zalophus californianus*; Fur seals: Ap = *Arctocephalus pusillus pusillus*, Ag = *Arctocephalus gazella,* Cu = *Callorhinus ursinus.*

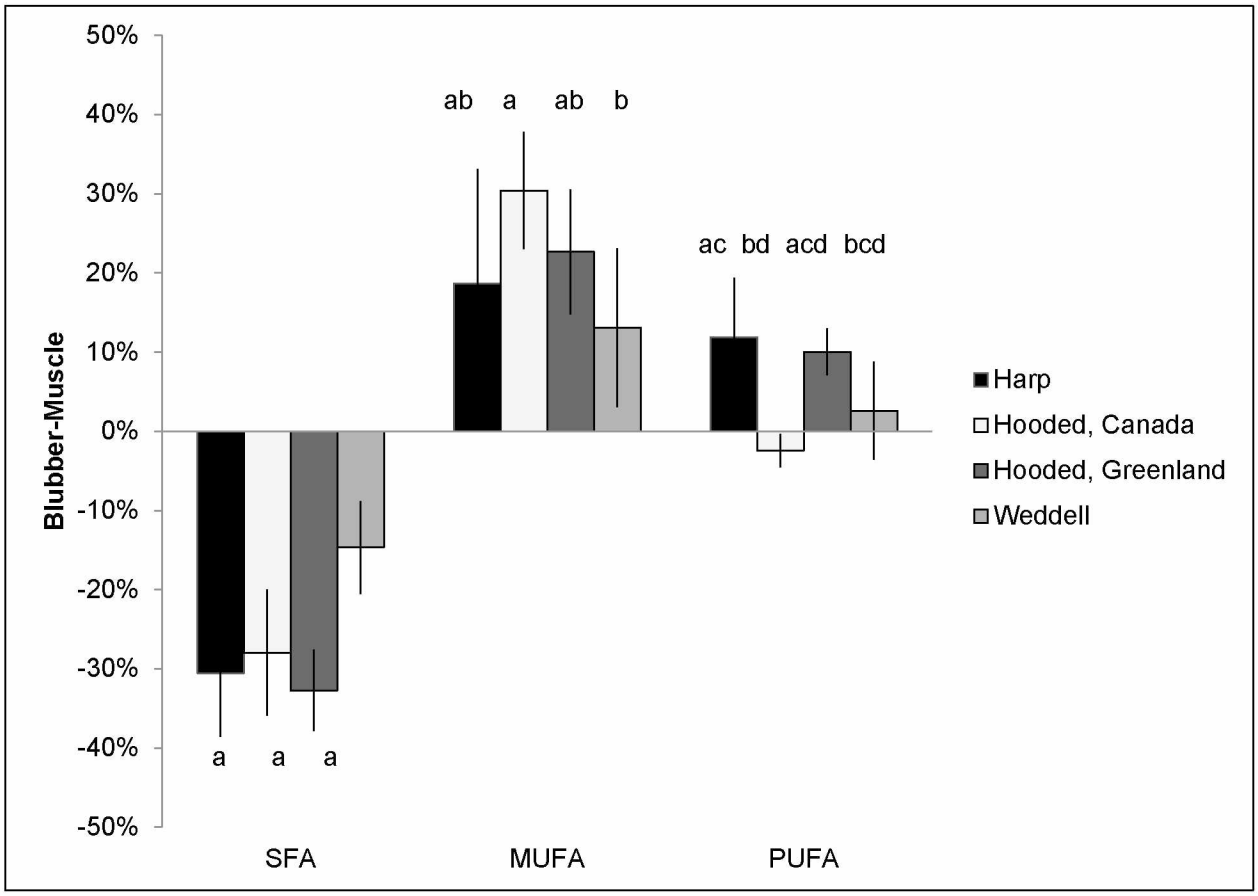

Figure 3.4: Difference between the relative proportion of each fatty acid class in blubber and muscle. Representing the relative proportion of each fatty acid class for harp, hooded (2 locations), and Weddell seals. Values > 0% indicate greater proportion of that particular FA class (SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid) in blubber; values < 0% indicate greater proportion in muscle. Different letters indicate significant differences ($p < 0.05$) among species within a fatty acid class.

Table 3.1: Body mass (kg) and latitude data. Data for otariids and phocids from the present study and published sources.

Species	Code	Sample size	Year	Age/sex class	Mass	Latitude	Mass reference	Fatty acid reference
Callorhinus ursinus	Cu	26	2007	adult female	40.0	48.54	Waite unpublished data	Waite et al. 2012
Arctocephalus pusillus pusillus	App	2		adult female	37.4	30.00	Arnould et al. 2005	Liwanag et al. 2012b
Arctocephalus pusillus pusillus	App	7	2002-2003	adult female	49.4	31.65	Arnould et al. 2005	Arnould et al. 2005
Arctocephalus pusillus pusillus	App	5	2003	adult female	42.0	19.45	Grahl-Nielsen et al. 2010	Grahl-Nielsen et al. 2010
Phocarctos hookeri	Ph	26	2000	adult female	55.0	50.86	Lambert et al. 2013	Meynier et al. 2014
Phocarctos hookeri	Ph	16	2007-2009	adult, both sexes	55.0	50.86	Lambert et al. 2013	Lambert et al. 2013
Arctocephalus tropicalis	At	2		adult female	35.0	62.51	Ferguson and Higdon 2006	Liwanag et al. 2012b
Arctocephalus gazella	Ag	2		adult female	37.4	37.83	Arnould 1995	Liwanag et al. 2012b
Eumatopius jubatus	Ej	6	2007	adult female	327.2	48.54	Waite unpublished data	Waite et al. 2012
Zalophus californianus	Zc	3		mixed, both sexes	81.0	37.14	Ferguson and Higdon 2006	Liwanag et al. 2012b
Erignathus barbatus	Eb	30	2003	mixed, both sexes	301.2	65.75	Krüger et al. 2014	Cooper et al. 2009
Erignathus barbatus	Eb	5	2002		301.2	71.30	Krüger et al. 2014	Budge et al. 2007
Cystophora cristata	Cc	5	2008	adult female	289.4	47.60	Present study	Present study
Cystophora cristata	Cc	9	1984	adult female	189.0	47.60	Iverson et al. 1995	Iverson et al. 1995
Cystophora cristata	Cc	19	2001	adult female	145.6	66.73	Present Study	Falk-Petersen et al. 2009
Cystophora cristata	Cc	19	2001	adult female	145.6	71.77	Present Study	Falk-Peterson et al. 2009
Cystophora cristata	Cc	4	2008	adult female	145.6	72.40	Present Study	Present study
Pusa hispida	Ph	29	1994-1997	mixed, both sexes	73.9	56.01	Krüger et al. 2014	Thiemann et al. 2008
Pusa hispida	Ph	23	2003		73.9	56.21	Krüger et al. 2014	Thiemann et al. 2007
Pusa hispida	Ph	27	1998, 2000	mixed, both sexes	73.9	62.98	Krüger et al. 2014	Thiemann et al. 2007
Pusa hispida	Ph	30	1992, 1998	mixed, both sexes	73.9	64.39	Krüger et al. 2014	Thiemann et al. 2007
Pusa hispida	Ph	15	2003	mixed, both sexes	73.9	65.75	Krüger et al. 2014	Cooper et al. 2009
Pusa hispida	Ph	7	1995, 2004	mixed, both sexes	73.9	70.33	Krüger et al. 2014	Thiemann et al. 2007
Pusa hispida	Ph	43	1996, 1999, 2001	mixed, both sexes	73.9	70.82	Krüger et al. 2014	Thiemann et al. 2007
Pusa hispida	Ph	4	2002		73.9	71.30	Krüger et al. 2014	Budge et al. 2007
Pusa hispida	Ph	39	2001, 2004	mixed, both sexes	73.9	71.89	Krüger et al. 2014	Thiemann et al. 2007
Pusa hispida	Ph	41	1998	mixed, both sexes	73.9	76.03	Krüger et al. 2014	Thiemann et al. 2007
Pusa hispida	Ph	42	1998	mixed, both sexes	73.9	77.42	Krüger et al. 2014	Thiemann et al. 2007
Halichoerus grypus	Hg	485	1993-2001	mixed, both sexes	172.5	43.55	Krüger et al. 2014	Beck et al. 2007
Halichoerus grypus	Hg	34	1996	adult female	185.0	56.17	Pomeroy et al. 1999	Walton et al. 2000
Halichoerus grypus	Hg	23	1996	adult female	172.5	59.10	Krüger et al. 2014	Walton et al. 2000
Phoca largha	Pl	24	2003	mixed, both sexes	78.7	65.75	Krüger et al. 2014	Cooper et al. 2009
Phoca vitulina	Pv	5	1998	adult female	75.6	48.97	Krüger et al. 2014	Smith et al. 1996
Phoca vitulina	Pv	16	1994-1995	adult, both sexes	75.6	57.63	Krüger et al. 2014	Smith et al. 1996
Phoca vitulina	Pv	8	1994-1995	adult, both sexes	52.1	57.79	Burns et al. 2005	Iverson et al. 1997
Phoca vitulina	Pv	14	1994-1995	adult, both sexes	52.1	58.13	Burns et al. 2005	Iverson et al. 1997
Phoca vitulina	Pv	63	1994-1995	adult, both sexes	52.1	60.22	Burns et al. 2005	Iverson et al. 1997
Phoca vitulina	Pv	9	1994-1995	adult, both sexes	52.1	60.79	Burns et al. 2005	Iverson et al. 1997
Phoca vitulina	Pv	10	1994-1995	adult, both sexes	52.1	60.79	Burns et al. 2005	Iverson et al. 1997
Phoca vitulina	Pv	5	1994-1995	adult, both sexes	83.2	78.33	Lydersen and Kovacs 2004	Andersen et al. 2004
Histriophoca fasciata	Hf	32	2003	mixed, both sexes	80.2	65.75	Krüger et al. 2014	Cooper et al. 2009
Pagophilus groenlandicus	Pg	5	2008	adult female	109.1	47.60	Present study	Present study
Pagophilus groenlandicus	Pg	12	2001	adult, both sexes	134.3	66.73	Falk-Petersen et al. 2009	Falk-Peterson et al. 2009
Lobodon carcinophaga	Lc	41	2001-2002	adult, both sexes	257.3	67.43	Burns unpublished data	Burns unpublished data
Leptonychotes weddellii	Lw	19	2003	adult female	393.1	77.85	Wheatley et al. 2007	Wheatley et al. 2007
Leptonychotes weddellii	Lw	9	2010-2012	adult female	444.1	77.85	Present study	Present study

Table 3.1 continued

Species	Code	Sample size	Year	Age/sex class	Mass	Latitude	Mass Reference	Fatty acid Reference
Mirounga angustirostris	Ma	15	2005	adult female	446.0	37.13	Fowler et al. 2014	Fowler et al. 2014
Mirounga leonina	Ml	11	2001	adult female	516.5	54.30	Krüger et al. 2014	Best et al. 2003
Mirounga leonina	Ml	27	1999	adult female	516.5	54.30	Krüger et al. 2014	Bradshaw et al. 2003

Latitude is reported as the absolute value in decimal degrees. 'Code' refers to species name abbreviation used in figures. Age class 'mixed' refers to adult, sub-adult, and/or juveniles present in the sample. Data were not available where no term for "Year collected" or "Age/sex class" is given.

Table 3.2: Results of linear mixed effects models. Models examined the correlations among latitude, body mass, and blubber thickness (tissue patterns only), and the relative proportions of fatty acid classes.

Patterns among 12 phocid species			
	FA class	Model	*p*-value
	PUFA	14.7200 + 0.1769(latitude)	0.038
	SFA	27.9600 - 0.2117(latitude)	< 0.001
Tissue patterns in harp, hooded, and Weddell seals			
	FA class	Model	*p*-value
Blubber	PUFA	0.7713 - 0.0940(log_mass)	log_mass: 0.001
	SFA	-0.3215 + 0.1095(log_mass) - 0.0245(blub_depth)	log_mass: < 0.001, blub_depth: 0.039
Muscle	PUFA	0.4045 - 0.0852(log_mass)	log_mass: 0.039
	MUFA	-1.7657 + 0.9303(log_mass) + 0.4392(blub_depth) - 0.1875(log_mass×blub_depth)	log_mass: 0.011, blub_depth: 0.016, interaction: 0.015
	SFA	1.7211 - 0.5517(log_mass) - 0.2806(blub_depth) + 0.1158(log_mass×blub_depth)	log_mass: 0.039, blub_depth: 0.038, interaction: 0.042

PUFA: polyunsaturated fatty acids; MUFA: monounsaturated fatty acids; SFA: saturated fatty acids. There were no significant models for monounsaturated fatty acids (MUFA) among the 12 phocid species models. latitude: absolute value in decimal degrees; log_mass: log(mass (kg)); blub_depth: blubber depth (cm).

Table 3.3: The relative proportions of each fatty acid class in blubber and skeletal muscle (mean ± 1 SD). Data from direct analyses of harp, hooded, and Weddell seal samples.

		Harp	Hooded (Canada)	Hooded (Greenland)	Weddell
Blubber	PUFA	33.53 ± 3.10 %[a]	19.09 ± 4.20 %[b]	33.84 ± 1.60 %[a]	19.42 ± 3.80 %[b]
	MUFA	55.44 ± 3.60 %[a]	67.75 ± 1.20 %[a]	53.17 ± 2.50 %[b]	55.20 ± 5.80 %[a]
	SFA	11.03 ± 0.80 %[a]	13.17 ± 1.20 %[a]	12.99 ± 1.60 %[a]	23.30 ± 5.60 %[b]
Muscle	PUFA	21.63 ± 5.60 %	21.54 ± 9.80 %	23.80 ± 3.10 %	16.81 ± 5.60 %
	MUFA	36.81 ± 12.00 %	37.35 ± 7.70 %	30.50 ± 8.70 %	42.12 ± 5.60 %
	SFA	41.56 ± 7.80 %	41.11 ± 8.10 %	45.70 ± 6.30 %	37.96 ± 9.50 %

Different superscripts indicate significantly different ($p < 0.05$) values from the other species for a given fatty acid class. PUFA: polyunsaturated fatty acid; MUFA: monounsaturated fatty acid; SFA: saturated fatty acid.

Table 3.4: Relative proportions of fatty acid classes in prey species. Published values of the fatty acid classes in the main prey species for harp, hooded, and Weddell seals.

Prey species	Common name	Predator	Total %ΣSFA	Total %ΣMUFA	Total %ΣPUFA	Location	Reference
Themisto spp.	Pelagic amphipod	Harp seal	11.7	70.9	15.4	Greenland Sea	Dahl et al. 2000
Themisto spp,			19.6	49.2	27.4	Barents Sea	Scott et al. 1999
Themisto spp.			12.5	84.1	4.2	Greenland Sea	Dahl et al. 2003
Mallotus villosus	Capelin	Harp seal, hooded seal	20.4	56.8	22.7	Greenland Sea	Dahl et al. 2003
Mallotus villosus			25.0	46.6	27.8	Greenland Sea	Dahl et al. 2000
Boreogadus saida	Arctic cod	Hooded seal	16.8	52.1	28.1	Greenland Sea	Dahl et al. 2000
Boreogadus saida			14.6	72.3	11.9	Greenland Sea	Dahl et al. 2000
Boreogadus saida			14.5	73.9	12.1	Barents Sea	Scott et al. 1999
Boreogadus saida			12.7	63.2	24.0	Greenland Sea	Dahl et al. 2003
Gonatus fabricii (adult)	Armhook squid	Hooded seal	6.7	78.2	15.1	Scotian Shelf	Hooker et al. 1999
Gonatus fabricii (juvenile)			22.8	30.6	46.6	Scotian Shelf	Hooker et al. 1999
D. mawsonii	Antarctic toothfish	Weddell seal	22.3	61.2	15.6	Ross Sea	Jo et al. 2013
P. antarcticum	Antarctic silverfish	Weddell seal	32.2	47.0	19.5	Ross Sea	Jo et al. 2013

%ΣSFA: relative proportion of saturated fatty acid; %ΣPUFA: relative proportion of polyunsaturated fatty acid; %ΣMUFA: relative proportion of monounsaturated fatty acid.

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Chapter 4: Seasonally persistent differences in the fatty acid profiles of female Weddell seal blubber and skeletal muscle[1]

Abstract

While it is well understood that the total amount of lipid stored in blubber and muscle of marine mammals is indicative of overall condition and nutritional status, the allocation of specific lipid classes among the main sites of lipid storage and their use is less well understood. We compared the fatty acids (FA) stored in blubber and muscle of adult female Weddell seals (*Leptonychotes weddellii*) to determine if FA composition differed between blubber, where lipids serve thermoregulatory and long-term energy storage roles, and skeletal muscle, where lipids are stored short-term prior to being oxidized to fuel work. We also investigated whether the FA profiles of blubber and/or muscle varied in response to seasonal shifts in body mass, body composition, reproductive status, and/or dive behavior. Overall, the blubber contained 15-20% more monounsaturated FA (MUFA; primarily 16:1 and 18:1) than muscle, while muscle contained ~15-50% more saturated FA (SFA) than blubber (primarily 16:0 and 18:0). Seals that were larger and/or had more total body lipid also had more 18:1*n*9 in their blubber, and 17:0 and 20:0 in muscle. The differences in FA profiles between muscle and blubber likely reflect blubber's primary function as insulation; MUFA remain fluid at lower temperatures, such as occur in the outer blubber layer, while SFA are dense sources of energy for muscle. Body mass was the most common predictor of the relative proportions of different FA classes in blubber and muscle as mass influences both thermodynamics and muscle metabolism.

Keywords

Blubber, fatty acids, metabolism, skeletal muscle, Weddell seal

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4.1 Introduction

In mammals, when energy intake is greater than energy demand, excess energy is stored primarily as white adipose tissue (WAT), and the total amount of lipid is generally indicative of an animal's overall condition and nutritional status (Young 1976; Pond 1978; Weber 2011; Champagne et al. 2012). Whereas most terrestrial mammals distribute excess energy among 12 internal adipose deposits, adult marine mammals store up to 75% of their lipid reserves as triacylglycerols (TAG) in their hypodermal blubber layer (Reilly and Fedak 1990; Pond and Ramsay 1992; Pond 1992; Shero et al. 2014). In addition to being the main energy reserve, this blubber layer acts as the primary source of insulation (Beck et al. 1993; Ryg et al. 1993; McDonald et al. 2008; Castellini et al. 2009; Liwanag et al. 2012a), aids in streamlining the body, and provides buoyancy (Webb et al. 1998; Beck et al. 2000; Biuw 2003). Reductions in the amount of lipid in the blubber layer may, therefore, cause increased metabolic costs due to increases in thermoregulation and cost of transport (Rosen et al. 2007).

Blubber is not the only tissue where lipid is deposited, though it contains the largest proportion of total body lipid. Large lipid droplets within the skeletal muscles can account for up to ~15% of muscle volume in phocids (Reed et al. 1994; Kanatous et al. 1999; Kanatous et al. 2008; Trumble et al. 2010). In contrast to lipids stored in the blubber, muscle lipid stores are immediately available to mitochondria for oxidation to ATP without the need for transport via the circulatory system (Connor et al. 1996; Dyck et al. 1997; Gurr et al. 2002). This localized supply is likely critical for deep diving marine mammals, because significant vasoconstriction limits blood flow to the peripheral organs and skeletal muscles while diving (Kooyman et al. 1981; Davis et al. 1991). In addition, the locomotory muscles of deep diving marine mammals consist primarily of oxidative (Type I and IID) fibers (Kanatous et al. 2008), and muscles composed of highly oxidative fibers are known to contain greater TAG stores than those that consist primarily of glycolytic fibers (Dyck et al. 1997; Gorski et al. 1999).

While energy balance is the primary influence on the amount of lipid stored within blubber and muscle, it likely has less of an influence on the individual lipids mobilized from each tissue (Raclot and Groscolas 1993; Raclot and Groscolas 1995; Raclot 2003; Mustonen et al. 2007a). When individual fatty acids (FA) are mobilized, the mobilization rate is unaffected by the relative amount of each FA until the supply of a particular FA is depleted (Ogawa et al. 1992; Raclot and Groscolas 1995; Kuroshima et al. 1995; Groscolas and Herzberg 1997; Raclot 2003).

The physiochemical properties and mobilization rates of FA are influenced by their degree of saturation and chain length. Longer, more unsaturated (i.e., more double bonds) FA are mobilized from tissue stores prior to shorter and more saturated forms (Raclot 2003). However, because net energy production declines with each double bond that must be broken prior to oxidation, saturated FA (SFA) produce more ATP per mole of FA consumed than monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA) (Gurr et al. 2002). Conversely, long-chain (> 18 carbon) FA with more double bonds consume fewer moles of oxygen to completely oxidize (Trumble and Kanatous 2012). In addition, the melting point of the lipid decreases as the number of double bonds increases, and SFA are solid at typical mammalian body temperatures (37 °C), while PUFA can be fluid at ambient temperatures as low as -49.5 °C (Irving et al. 1957; Gurr et al. 2002). In pinnipeds, diet is the primary determinant of tissue lipid profiles (Dalsgard 2003; Iverson et al. 2004). However, ingested FA can be modified by elongation, shortening, desaturation, and endogenous *de novo* synthesis (Budge et al. 2006), and tissue lipid profiles may diverge from dietary intake in response to physiochemical properties of the lipids that influence their physiological use (Weber 1992; Raclot 2003; Weber 2011; Trumble and Kanatous 2012).

As a result of the differences among FA classes (i.e., SFA, MUFA, PUFA), tissues that act as long-term energy stores may contain a greater proportion of FA that are unsaturated and/or have longer chain lengths, which are easily mobilized and transported. In contrast, tissues that oxidize FA quickly may contain more energy-dense FA that are saturated and/or have shorter chain lengths. The muscles of deeper diving individuals may contain disproportionately higher levels of PUFA to maximize ATP production when circulation is restricted and oxygen is limited (Castellini et al. 1992; Trumble and Kanatous 2012). However, thermoregulatory constraints may result in higher proportions of unsaturated fatty acids in tissues exposed to colder temperatures than would be predicted based on energetics alone (Irving et al. 1957; Sokolov 1962; Raclot and Groscolas 1993; Raclot and Groscolas 1995; Connor et al. 1996; Vaillancourt and Weber 2007; Vaillancourt et al. 2009). For example, the outer blubber layer in bottlenose dolphins (*Tursiops truncatus*) is dominated by MUFA during summer, but the proportion of PUFA increases during winter as water temperature decreases (Samuel and Worthy 2004). In addition, for a given temperature regime, the proportion of SFA may be lower and PUFA higher, in individuals with less lipid stores (lower body condition) (Irving et al. 1957; Liwanag et al.

2012b). Seasonal shifts in FA profiles within any given tissue may occur because of variation in diet, lipid intake, and use rates, which can ensue during periods of mass gain or loss (Castellini et al. 1985; Zhao et al. 2006; Sparling et al. 2006; Beck et al. 2007; Atkinson et al. 2008; Rea et al. 2009). For example, many phocids gain considerable body mass and lipid mass during the winter foraging period (Webb et al. 1998; Aoki et al. 2011; Costa et al. 2012). If the winter diet differs from the summer diet, or if FA biosynthesis and/or sequestration rates differ during periods of caloric excess, tissue FA profiles would likewise exhibit seasonal differences (Mustonen et al. 2007b). Further, lactating pinnipeds preferentially mobilize long-chain MUFA and some PUFA from blubber into milk, and this alters their blubber lipid profile relative to females that are not lactating (Iverson et al. 1995; Mellish et al. 1999a; Wheatley et al. 2008; Fowler et al. 2014) .

This study compares the FA composition in blubber and muscle of adult female Weddell seals (*Leptonychotes weddellii*) to understand lipid dynamics in a large, lipid-dependent, seasonally-active polar phocid. We examine if temporal, physiological, and behavioral factors influence the FA composition of each tissue. We hypothesize that as a result of cold ambient temperatures, blubber will be dominated by a greater relative proportion of MUFA and PUFA, and seals in better body condition (greater total body lipid: TBL) will have less PUFA than thinner seals, as the thick blubber reduces the steepness of the gradient between ambient and core temperatures. Further, we hypothesize that the muscle may contain greater SFA, as it provides a dense energy source. Alternatively, the need to conserve oxygen while diving will result in a reduced SFA content in muscle as compared with blubber, with a lower proportion of SFA in muscle of individuals that made the longest/deepest dives. Finally, we predict that seals that regain the most body mass and lipid over the winter will have the largest seasonal difference in the FA profile in each tissue, gaining more MUFA in blubber and PUFA in muscle, as they deposit the most new energy stores.

4.2 Methods

4.2.1 Sample collection

Adult female Weddell seals were captured in Erebus Bay (~77°S, 165°E), and along the Victorialand coastline (~76°S, 162°E) of the Ross Sea, Antarctica in the austral fall (January/February; $n = 43$) and spring pupping period (October/November; $n = 33$) from 2010 to

2012. Reproductive history and other demographic data were obtained for individual seals from a collaborating research group (Project B009 lead by J. Rotella and R. Garrott). Fifteen of the 76 individuals were handled in fall and the following spring of the same calendar year, and these individuals were used for seasonal comparisons. All spring seals were sampled during the pupping period, but only 13 of the 33 females had pupped. For lactating females, all data were collected within five days of giving birth. Of the 43 animals handled in fall, all were fully molted, 23 were known to have skipped pupping the previous spring, and the remaining 20 were assumed to have skipped pupping based on their molt status (in a larger study, < 15% of fully molted females had pupped earlier in the year; Burns et al. 2013). Thus, all study animals fall within three categories: post-molt/skip breeding (fall); lactating (spring); and non-reproductive (spring).

All seals were anesthetized with an intramuscular injection of 1.0 mg kg^{-1} tiletamine/zolazepam HCl, followed by intravenous injections of ketamine/diazepam (1:1 ratio, 100 mg ml^{-1} and 5 mg ml^{-1}) as necessary to maintain sedation (0.20 mg kg^{-1} ketamine, ~0.01 mg kg^{-1} diazepam). Muscle (*Longissimus dorsi*) and full core blubber biopsies were taken from the posterior flank of each seal. The biopsy site was prepared by injecting 1 ml lidocaine subcutaneously, trimming the hair, and scrubbing the site with sterile gauze and 10 % providone-iodine solution (Betadine®, Stamford, CT, USA). A 1 cm incision was made in the epidermis and dermis using a scalpel prior to tissue collection. Blubber (full-thickness) and muscle biopsies were collected separately using a 6 mm biopsy punch (blubber) and a biopsy cannula (muscle). Biopsy samples were flash frozen in liquid nitrogen, and stored at -80 °C until analysis.

Total body mass (± 0.5 kg) was determined by direct weighing (MSI-7200-IT Dyna-Link digital dynamometer, Measurement Systems International, Seattle, WA, USA), and TBL was determined by isotopic dilution (Shero et al. 2014). If body mass (n = 3) or body condition (n = 5) data were unavailable for any particular animal, values were estimated based on morphometric measurements (Shero et al. 2014). As part of a separate study, all females handled in fall were equipped with a satellite-linked CTD recorder (Sea Mammal Research Unit, St. Andrews, Scotland) that recorded a suite of behavioral and environmental variables (Goetz et al. 2010). Because average dive duration was bimodally distributed and significantly correlated with female mass (Shero 2015), when testing for the effect of behavior on FA profiles, we only considered dives > 3 min long (this produced a unimodal distribution) and used the residual from

the mass-dive duration correlation, rather than the actual dive duration. Dive parameters for animals in the fall sampling period were calculated from dives made in the first 8 weeks following sample collection.

4.2.2 Fatty acid analysis

Samples of blubber (0.200 - 0.500 g) and muscle (0.030 - 0.120 g) were thawed and weighed to the nearest 0.001 g (wet mass). Lipid was extracted in chloroform: methanol 2:1 (Folch et al. 1957) on a Soxhlet apparatus. Fatty acid methyl esters (FAME) were prepared as described by Budge et al. (2006). Blubber and muscle from 32 females (n = 23 Jan; n = 9 Oct) were analyzed on a Varian 3900 GC-FID (Varian Inc., Walnut Creek, CA, USA) at Baylor University (Waco, TX, USA; referred to as Baylor throughout) following protocols of Budge et al. (2006) with the following modifications: Column CP-Select for FAME (CP419) 100 m × 0.25 mm ID × 0.25 μm. The injector temperature was 250 °C with a 1 μl injector split ratio of 50:1. Column flow was 1.0 ml min^{-1} programmed at 210 °C for 9.0 min and ramped at 15 °C min^{-1} to 260 °C for 7.7 min. Detector temperature was set at 300 °C with a hydrogen flow of 30 ml min^{-1} and airflow of 300 ml min^{-1}. Due to instrument problems, the remaining 37 blubber and muscle samples (n = 15 Jan; n = 22 Oct) were analyzed on a Hewlett Packard 5890 Series II Plus GC-FID (Hewlett-Packard, Palo Alto, CA, USA) at the ASET Laboratory at the University of Alaska Anchorage (referred to as ASET throughout), following the same protocols. The column was an Agilent Technologies DB-23 60 meter × 0.25mm ID × 0.25 μm. Injector temperature was 125 °C. Column flow was 1.0 ml min^{-1} and ramped at 3 °C min^{-1} to 240 °C for 1.67 min. At both Baylor and ASET, individual FA concentrations in the sample were determined from a 5-point standard curve created using the Supelco 37 Component FAME Mix (Sigma-Aldrich Co., St. Lewis, MO, USA). The standard curve included a range of concentrations representative of FA in the samples analyzed. Chromatograms from GC-FID analysis were manually verified for correct peak identification and integration. FA identities were verified via mass spectroscopy (Hewlett Packard 6890 GC and 5973 Mass selective detector; Hewlett Packard, Palo Alto, CA, USA) in four samples (two blubber, two muscle). Because samples were completely consumed by analysis, only seven samples (four muscle, three blubber) were analyzed in both laboratories. We detected 31 of the 37 FA used as standards in both blubber and muscle. Both laboratories identified the same 14 FA at above trace amounts (> 0.5%) in blubber, and the same 28 FA at

above trace amounts (> 0.5%) in muscle. FAME were described by shorthand nomenclature: [carbon number]:[number of double bonds]*n*[position of first double bond counted from the methyl end] according to the IUPAC (IUPAC-IUB Commission on Biochemical Nomenclature 1976). All FA data are presented as percent contribution by weight of the particular FA to the sum total of all FA detected in sample (mean ± 1 SD). Relative proportions of fatty acids by class were determined by summing the percent of all identified FA within each class (where %Σ indicates the relative proportion of saturated [%ΣSFA], monounsaturated [%ΣMUFA] or polyunsaturated [%ΣPUFA] FA).

4.2.3 Statistical analysis

All proportional data were arcsine-square root transformed prior to analysis, and all analyses were completed in SPSS (v 21, IBM, Armonk, NY, USA) or the R package (v 3.1.2, R Development Core Team, 2014). Differences were considered significant at $p < 0.05$, and all values are reported as mean ± 1 SD.

For the seven samples run in both laboratories, the between-lab differences in the relative proportions of FA classes and individual FA were assessed using paired t-tests with Bonferroni correction. As there were significant lab effects, lab was retained as a factor in all analyses, and the overall conclusions are based on results from both analytical laboratories.

We used MANOVAs with Bonferroni correction to determine if there were differences in the $\%\Sigma FA_C$ (where FA_C represents a FA class; %ΣSFA, %ΣMUFA, %ΣPUFA), and individual FA ($\%FA_i$) between reproductive (n = 13) and non-reproductive (n = 20) females in spring in blubber or muscle. As there were no significant differences by reproductive status, groups were combined for all subsequent analyses. The difference in $\%\Sigma FA_C$ and $\%FA_i$ between blubber (B) and muscle (M) samples (ΔFA_{B-M}) was calculated for each seal. We then tested for seasonal differences in ΔFA_{B-M} for $\%\Sigma FA_C$ and $\%FA_i$ using a MANOVA with Bonferroni correction. As there were no significant seasonal differences, fall and spring samples were combined prior to testing if the mean ΔFA_{B-M} was significantly different from zero for each $\%\Sigma FA_C$ and $\%FA_i$ (t-test). The $\%FA_i$ cauesd the greatest disparity within each analytical lab as identified by SIMPER (similarity percentages) analysis. The SIMPER procedure compares the mean abundance and examines the contribution of each FA to the mean Bray-Curtis dissimilarity between two defined groups (e.g., blubber and muscle). SIMPER analyses were conducted using the VEGAN package in R (Oksanen et al. 2015). We used forward stepwise regression models to investigate if

physiological or behavioral factors influenced the $\%\Sigma FA_C$ and $\%FA_i$ of muscle or blubber, with reproductive status (repro status), body composition (TBL), body mass (mass), analytical lab (lab), and season as covariates. Due to sample size limitations for several of the covariates (reproductive status, season) by lab, both labs were included in a single model rather than run as separate models for comparison. Body mass was log transformed to approximate normality prior to inclusion in the models. Because dive data were only available for 39 of 76 females, a second set of models that included dive duration residuals (dive resid) in addition to the covariates listed above were run. Second-order Akaike information criterion tests (AICc) were used to select the best model, and variables were only included if the ΔAICc of the added parameter was < 2 (Burnham and Anderson 2002). Models where analytical lab was the only significant predictor of FA profiles are not reported. For model results, where season is a significant parameter, the coefficient is multiplied by "0" for January, and "1" for October. Where lab is a significant predictor, the coefficient is multiplied by "0" for ASET, and "1" for Baylor.

The effect of season on the relative proportions of FA ($\%\Sigma FA_C$ and $\%FA_i$) of blubber ($n = 11$) and muscle ($n = 8$) was tested by calculating the difference in FA proportions between January and October (ΔFA_{J-O}) for samples from females that were handled in both seasons, and where both samples were assayed in the same lab. The mean difference was compared to zero using a t-test, and stepwise regression models were used to test for significant physiological or behavioral effects. Covariates included analytical lab (lab), reproductive status in spring (repro status), fall mass (Jan mass), fall TBL (Jan TBL), spring TBL (Oct TBL), the overwinter change in mass and TBL (TBL change), and fall dive duration residual. Body mass was log transformed to improve normality prior to inclusion in the models. Significance ($p < 0.05$) and model selection criteria were as described above.

4.3 Results

We detected 31 of the 37 FA used as standards in both blubber and muscle. All FA found in blubber were also present in muscle (Table 4.1). Because we were primarily interested in between-tissue differences, all 28 FA detected above trace levels ($> 0.5\%$) in muscle were included in analyses for both tissues. There were significant differences by analytical lab in each $\%\Sigma FA_C$ and $\%FA_i$ in the seven-sample subset run in both labs ($p < 0.05$ in all cases). Yet, both

labs identified the same classes and individual FA as the most abundant within each tissue (Table 4.1), and the overall patterns in ΔFA_{B-M} of both $\%\Sigma F_C$ and $\%FA_i$ were similar.

Overall, ΔFA_{B-M} was significantly different from zero for 25 (ASET) and 21 (Baylor) of the 28 FA detected ($p < 0.05$ in all cases). Results from both labs indicated that %ΣSFA was 14% (ASET) or 19% (Baylor) greater in muscle than blubber ($p < 0.001$; Figure 4.1A), largely driven by a 4-8% greater relative proportion of 16:0 (both labs: $p < 0.001$), and a 5-12% greater relative proportion of 18:0 in muscle (both labs: $p < 0.001$; Figure 4.1B). %ΣMUFA was 13% (ASET) or 22% (Baylor) higher (both labs: $p < 0.001$) in blubber than muscle (Figure 4.1A), due to greater proportions of 16:1*n*7 (5-11%; both labs: $p < 0.001$) and 18:1*n*9 in blubber (8-9%; both labs: $p < 0.001$; Figure 4.1B). SIMPER analysis showed the same four FA (16:0, 18:0, 16:1*n*7, and 18:1*n*9) contributed to the dissimilarity between tissues, combined accounting for 13.9% (ASET) or 20.8% (Baylor) of the dissimilarity. Specifically, 16:1*n*7 accounted for 3.1% (ASET) or 5.7% (Baylor; $p = 0.001$ both labs) of the dissimilarity between tissues, 18:1*n*9 accounted for 4.9% (ASET) or 5.4% (Baylor, $p = 0.001$ both labs), 18:0 accounted for 3.0% (ASET) or 5.3% (Baylor; $p = 0.001$ both labs), and 16:0 accounted for 2.8% (ASET) or 4.3% (Baylor; $p = 0.001$ both labs). Overall, there was no significant difference in %ΣPUFA between the two tissues for either lab (Figure 4.1A). However, many individual PUFA, such as 20:2*n*6, 20:3*n*6, and 20:4*n*6, were present in significantly greater relative proportions in muscle than blubber, though the difference between tissues was generally less than 1% (p-values all < 0.010; Figure 4.1B). The exception was 22:6*n*3, which made up a significantly greater relative proportion in blubber than muscle (both labs: $p < 0.001$).

In blubber, none of the physiological or behavioral variables measured (e.g., mass, TBL, reproductive status, dive residual) accounted for a significant amount of variation in the proportion of %ΣSFA, %ΣMUFA, or %ΣPUFA. However, for a few $\%FA_i$, models including analytical laboratory, body mass, season, and/or TBL accounted for substantial variability (R^2 ranging from 0.59 – 0.90; Table 4.2), although only 10-15% of the variability could be attributed to physiological parameters. In general, heavier (greater mass) seals had less 16:0 ($p = 0.049$), 14:1*n*5 ($p < 0.001$), and 18:2*n*6 ($p < 0.001$), and more 18:1*n*9 ($p = 0.032$) in blubber than did lighter individuals. Dive duration residual was not a significant predictor in any model in which it was included.

In muscle, none of the physiological or behavioral covariates accounted for significant variability in %ΣSFA, %ΣMUFA, or %ΣPUFA. In addition, while analytical lab and physiological factors did account for some of the measured variation in seven $\%FA_i$ (three SFA, two MUFA, two PUFA; Table 4.2), the amount of variation explained in muscle was smaller than in blubber (4 – 54%), with no more than 5% of the variability attributable to physiological parameters; Table 4.2). Dive duration residual was not a significant predictor in any model in which it was included.

There were few seasonal changes, but it is noteworthy that there was individual variation in blubber lipid profiles obtained from the same seals captured in both fall and spring (Figure 4.2). Among SFAs, the relative proportions of 14:0, 16:0 were 6.1% and 9.2% higher in blubber, respectively, in spring compared with fall (both $p < 0.001$), whereas the relative proportion of 14:1*n*5 was 7.9% higher in fall ($p < 0.001$). There were no seasonal differences in blubber %ΣMUFA or %ΣPUFA, but the relative proportion of 20:3*n*3 was 3% higher ($p = 0.025$), and 22:2*n*6 was ~1% higher ($p = 0.045$) in blubber in spring compared with fall.

In blubber, no physiological or behavioral covariates accounted for significant variability at the FA class level. However, mass and condition were predictors of seasonal differences in several $\%FA_i$ in blubber. In general, lighter females in fall had less seasonal difference in two SFA (20:0 and 22:0), two MUFA (17:1*n*7, and 22:1*n*9), and two PUFA (18:2*n*6 and 20:2*n*6) though the effect was negligible (Table 4.3). TBL change was a predictor of seasonal change, where seals with greater TBL had a 0.77% increase in $\Delta FA_{J\text{-}O}$ of 22:1*n*9 for every 1% increase in TBL. Additionally, a 1% increase in TBL was associated with 1.02% and 1.26% increases in $\Delta FA_{J\text{-}O}$ of C18:3*n*3 and C22:2*n*6, respectively. Reproductive status was a significant predictor of the seasonal variation in three FA (14:1*n*5, 21:5*n*3, and 22:5*n*3; Table 4.3), and the two PUFAs that were more abundant in blubber of non-reproductive animals than lactating ones, contributed < 2% to the total proportion of blubber FA. Non-reproductive females in spring had smaller seasonal changes in 14:1*n*5 than reproductive females, but they also showed increases in 21:5*n*3 and 22:5*n*3. Overwinter changes in the proportion of two PUFA (18:3*n*3 and 22:2*n*6) in the blubber were 1.5% and 2.5% lower (Table 4.3) in females that dove longer than expected based on their body mass.

There were no seasonal differences in any lipid class in muscle (Figure 4.2), nor could any significant proportion of the variability in %ΣSFA, %ΣMUFA, and %ΣPUFA be accounted

for by anything other than the analytical laboratory. However, for $\%FA_i$, analytical lab and physiological factors accounted between 71% and 97% of the variation in the over winter change in $\%FA_i$ (Table 4.3), although only 15–20% of the variation was due to physiological factors. Body condition (TBL) influenced over winter changes in three FA (16:0, 18:2*n*6, and 23:0), though the effect, while significant, was small and the direction of the effect varied by FA. Female mass in fall, but not in spring, or change in mass, was the most frequent predictor of over winter changes in the relative proportions of muscle FA (Table 4.3) though the effect was generally small. The directions of the correlations observed in muscle were frequently opposite to that seen in blubber (Table 4.3). Dive behavior was never a significant factor in models of the relative abundance of any individual FA in muscle.

4.4 Discussion

We found that fatty acid profiles differed between blubber and muscle; these differences persisted across seasons, were independent of female reproductive state, and may represent differential allocation of FA between the two tissues. Two physiological parameters were consistently significant predictors of $\%FA_i$ in each tissue: body mass and TBL. While the magnitudes of these effects were often small, these findings suggest that the FA profiles of blubber and muscle reflect their roles within the body. Blubber contained a greater proportion of MUFA, which remain fluid at lower temperatures, while the muscle contains a larger proportion of SFA, which produce the greatest amounts of ATP per mole oxidized to support metabolism. These differences were primarily driven by changes in the relative proportions of the four most abundant FA, 16:0 and 18:0, and their monounsaturated derivatives, 16:1*n*7 and 18:1*n*9. Together, these four FA represent ~55% of the total FA in blubber, and 49% of the total FA in muscle.

4.4.1 Variation in tissues

There were differences in the relative proportions of FA classes and individual FA in blubber and muscle in all females handled, regardless of reproductive status, in both seasons. Contrary to our hypothesis that lighter females would possess greater %ΣPUFA in blubber because of thermal constraints, 18:2*n*6 was the only PUFA in the blubber for which mass was a predictor. Heavier females tended to have relatively more 18:1*n*9 and 14:1*n*5, and less 16:0 in

their blubber than in muscle, whereas the opposite was true of lighter females. While the difference between heavier and lighter seals could reflect differences in diet, it might also indicate heavier females or those with greater total body lipid could better allocate lipid between tissues to meet thermoregulatory and metabolic needs (Wheatley et al. 2008). For example, heavier lactating females were able to extend lactation length and increase energy transfer versus lighter lactating females (Wheatley et al. 2006). Regardless, maintaining a high %ΣMUFA in blubber ensures that seals are able to maintain the flexibility of blubber while diving in cold water. Liwanag et al. (2012b) found positive correlations between the thermal conductivity of blubber and the relative proportions of individual long-chain PUFA (22 carbon chain length), whereas there was a negative correlation between conductivity and MUFA (16:1*n*7–11 and 17:0 in particular). Few individual FA within a class may contribute to a tradeoff between blubber flexibility and thermoregulation for an individual. The influence of mass on $\%FA_i$ in blubber may relate to the thermodynamics associated with increasing mass. Larger or heavier animals have a reduced area for heat loss, and the ability to naturally carry more blubber than smaller or lighter animals. Further, mass and blubber thickness were shown to be the most important influences on heat loss both via radiation and conductive pathways in Weddell seals (Mellish et al. 2014).

Saturated FA are less soluble than MUFA and PUFA (Gurr et al. 2002). Directly depositing SFA in muscle rather than blubber would facilitate their use as fuel when blood flow is restricted. The lower proportions of long-chain SFA compared with shorter chain SFA (20:0, 21:0, and 22:0 versus 18:0 and 16:0) further highlights the use of SFA as fuel for diving activities, as the longer chain SFA would be mobilized first based on the pattern of FA mobilization when longer chain FA are mobilized first (Raclot 2003). Trumble et al. (2010) also showed SFA were the second largest FA component after MUFA in Weddell seal skeletal muscle. PUFA represented the smallest FA class proportion in both tissues, and total PUFA content did not differ between tissues. However, the relative proportion of 20:5*n*3 in both blubber and muscle of Weddell seals (0.5% in blubber, and 1.3% in muscle) was lower than in their main prey (7% in *Pleuragramma antarcticum* and *Dissostichus mawsoni*; Burns et al. 1998; Plötz et al. 2001; Lenky et al. 2011). This may suggest preferential mobilization of this particular FA for other fates within the body, as it is important in brain function (Lauritzen et al. 2001) and highly mobilized into milk (Jump 2002). Seal skeletal muscle preferentially oxidizes PUFA

while diving to conserve oxygen, and diets with high %ΣPUFA may improve foraging efficiency through changes in metabolic costs while underwater (Fahlman 2012; Trumble and Kanatous 2012). However, in this study, dive residual was not a predictor of the proportion or seasonal change in any PUFA (or any other individual FA) in muscle. In addition, longer chain PUFA were present in greater proportion in skeletal muscle than shorter chain PUFA (Table 1), contrary to what would be expected if PUFA were being mobilized for oxidation based on chain length and degree of unsaturation (Raclot and Groscolas 1993; Raclot 2003). However, without specific metabolic tracer studies in seals of SFA and PUFA use in muscle while diving, the specific mobilization patterns are unknown.

4.4.2 Seasonal variation within tissues

While the difference in FA profiles between muscle and blubber (ΔFA_{B-M}) was consistent among individuals, the magnitude and direction of the seasonal variation in the FA profile of a given tissue (ΔFA_{J-O}) differed by individual. While this variation obscured differences at the FA class proportion (%ΣPUFA, %ΣMUFA, %ΣSFA), a significant portion of the variability in $\%FA_i$ was accounted for by the measured physiological and behavioral traits. For example, different $\%FA_i$ exhibited seasonal changes between lower body mass animals or those in worse body condition (TBL) than heavier animals or those in better body condition. Heavier animals had relatively lower proportions of PUFA (18:3*n*3, 20:2*n*6) in their blubber in spring than in fall. Seasonal changes based on animal size are likely a result of seasonal changes in TBL, which accompany reductions in diving and foraging effort during the reproduction and molting periods (Stirling 1969; Castellini et al. 1985; Boyd et al. 1993; Arnbom et al. 1993; Bennett et al. 2001; 2007 Sparling et al. 2006). Change in body condition prior to the over winter foraging period appear to supersede any mass or condition regained over the winter, as mass regained over the winter was not a predictive factor of class or individual FA proportions of blubber or muscle.

Changes in FA mobilization within tissues relating to phenology have been previously documented in pinnipeds (Wheatley et al. 2008; Arriola et al. 2013; Fowler et al. 2014; Miller 2014). Lactating gray (*Halichoerus grypus*), northern elephant (*Mirounga angustirostris*), and Weddell seals selectively mobilize blubber MUFA (20:1*n*9, C22:1*n*9) and PUFA (20:5*n*3, 22:6*n*3) into milk (Wheatley et al. 2008; Arriola et al. 2013; Fowler et al. 2014), which results in a reduction of these FA in the blubber layer over the course of lactation (Iverson et al. 1995; Iverson et al. 1997; Mellish et al. 1999b; Fowler et al. 2014). Contrary to these expectations, we

did not find any significant difference between the relative proportions of FA in blubber or muscle of reproductive and non-reproductive females in October. This suggests that five days may be too short for lactation-induced changes in the blubber FA profile of Weddell seals. Indeed, when changes in the FA composition of Weddell seal blubber over lactation were quantified, blubber FA of females sampled 1–6 days post parturition were considered baseline FA quantities (Wheatley et al. 2008). The long lactation period of Weddell seals (33-52 days; Hill 1987; Wheatley et al. 2006) likely accounts for the slow change in blubber FA (Wheatley et al. 2008). Though reproductive status was a predictor of seasonal variation of three FA in blubber (14:1*n*5, 21:5*n*3, 22:5*n*3), any costs associated with gestation, and the subsequent lactation period, appear to have little effect on over winter changes in blubber FA.

The proportions of six muscle FA were influenced by TBL and one by mass, where fatter (greater TBL) and heavier seals had greater proportions of two SFA and two PUFA, and lower proportions of two MUFA than leaner, lighter seals. In contrast, seasonal changes in muscle fatty acids within an individual were best explained by an individual's mass at the initial handling (spring mass), but not over winter changes in body mass or TBL, though this effect was often small as inter-lab differences dominated. In general, females that were lighter in fall, tended to gain more FA in all classes than heavier individuals. Because this is not the same pattern as observed in blubber, it is unlikely that these differences represent changes in diet, but instead likely represent differences in seasonal activity patterns and reliance on lipid reserves among animals of varying size. Smaller animals may be allocating resources to self-maintenance more so than larger individuals (Wheatley et al. 2006). Wheatley et al. (2006) further suggested foraging success and habitat use varied between reproductive animals of varying mass, whereas smaller animals may be more susceptible to environmental variation. This may not only be the case for reproductive animals, but all small individuals. Even though lipids are the main metabolic fuel year-round, reduced foraging while lactating or molting requires seals to rely on endogenous lipid stores to fuel metabolism (Champagne et al. 2012). This may be an especially energetically demanding time for lighter animals. The subsequent winter foraging period, when animals improve in body condition and store lipid in both muscle and blubber, may be especially important for lighter animals to regain specific FA stores and allocate them to either muscle or blubber.

Overall, physiological traits that influence metabolism, such as body mass, had a greater influence on the FA composition of blubber and muscle than the behavioral predictor, dive duration residual. However, inter-lab differences masked these effects, and made the definite strength of these correlations difficult to assess. In general, the physiological predictors and the allocation of FA between tissues fit with the main roles of each tissue in the body. Body size and TBL directly affect thermoregulation, thus, it follows that this would be an important predictor of the FA composition of the blubber layer. This may be driven by differences in relatively few individual FA, as only four FA were responsible for a large proportion of the difference between the blubber and muscle observed here. Body mass affects muscle metabolism and rates of oxygen use, which in turn directly affect dive duration and an animal's foraging effort (Kanatous et al. 1999; Falhman et al. 2012). Further, lighter seals may have different over winter activity patterns than larger animals (Wheatley et al. 2006), which is reflected in the FA gained and stored in both muscle and blubber. While we did not find any clear correlations between dive duration on the FA profiles of muscle or blubber, there may be other factors involved, while diving (i.e., depth, diet, foraging efficiency) that may account for the different patterns of FA allocation and seasonal effects between small and large animals.

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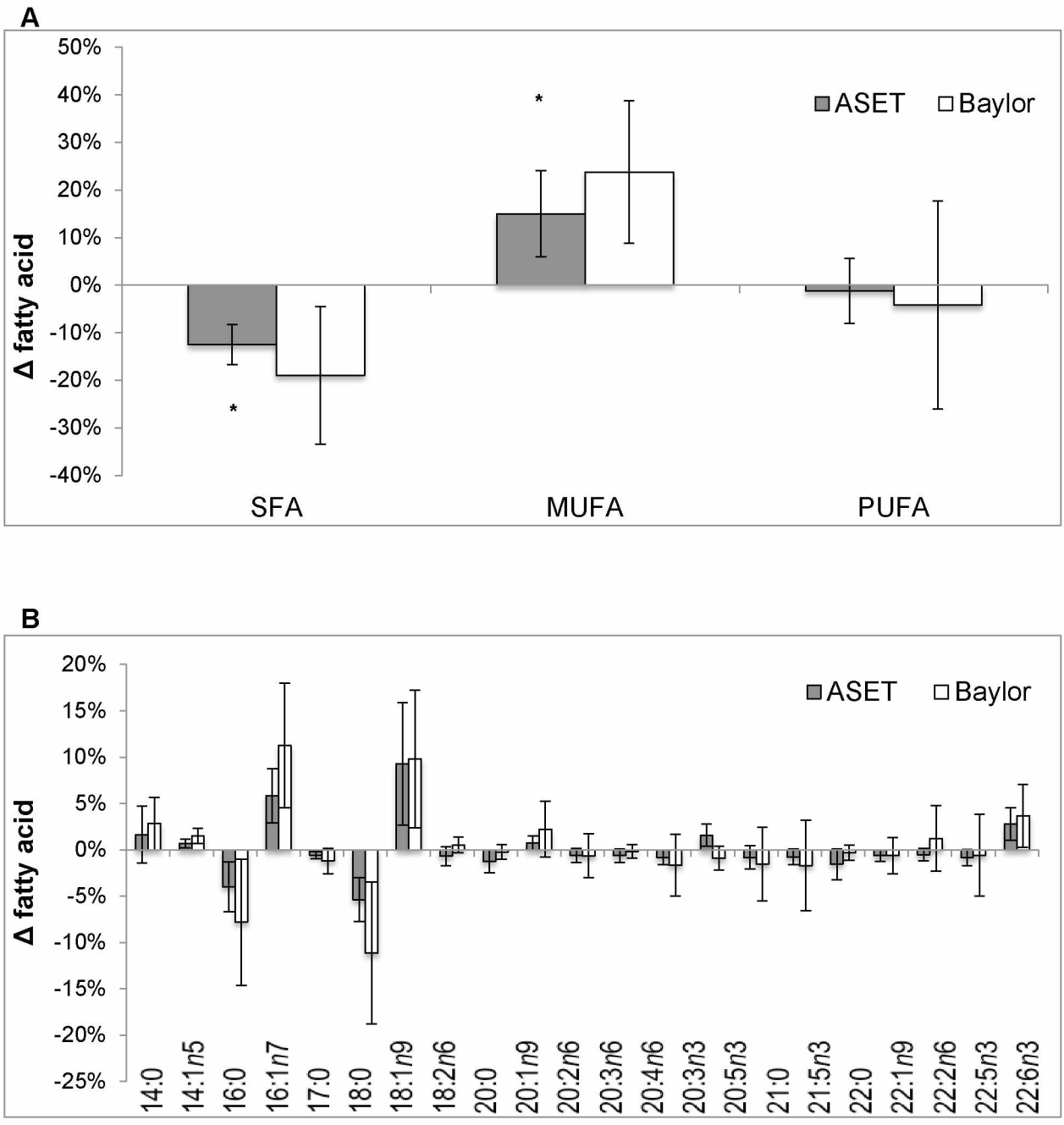

Figure 4.1: Mean ± 1 SD difference between blubber and muscle ($\Delta FA_{B\text{-}M}$). Relative proportion of fatty acid class (A) and individual fatty acids (B) for all adult female Weddell seals. Values > 0% represent a greater relative proportion of a fatty acid present in blubber, while values < 0% represent a greater relative proportion of a fatty acid present in muscle. (A) * indicates $\Delta FA_{B\text{-}M}$ (ASET) significantly different from 0 ($p < 0.05$). Sample size: $n = 37$ ASET, $n = 32$ Baylor. (B) All FA differences are significantly different from 0 ($p < 0.05$). While 26 fatty acids had $\Delta FA_{B\text{-}M}$ significantly different from 0, for visual clarity, only fatty acids with a $\Delta FA_{B\text{-}M} > 0.5\%$ are shown.

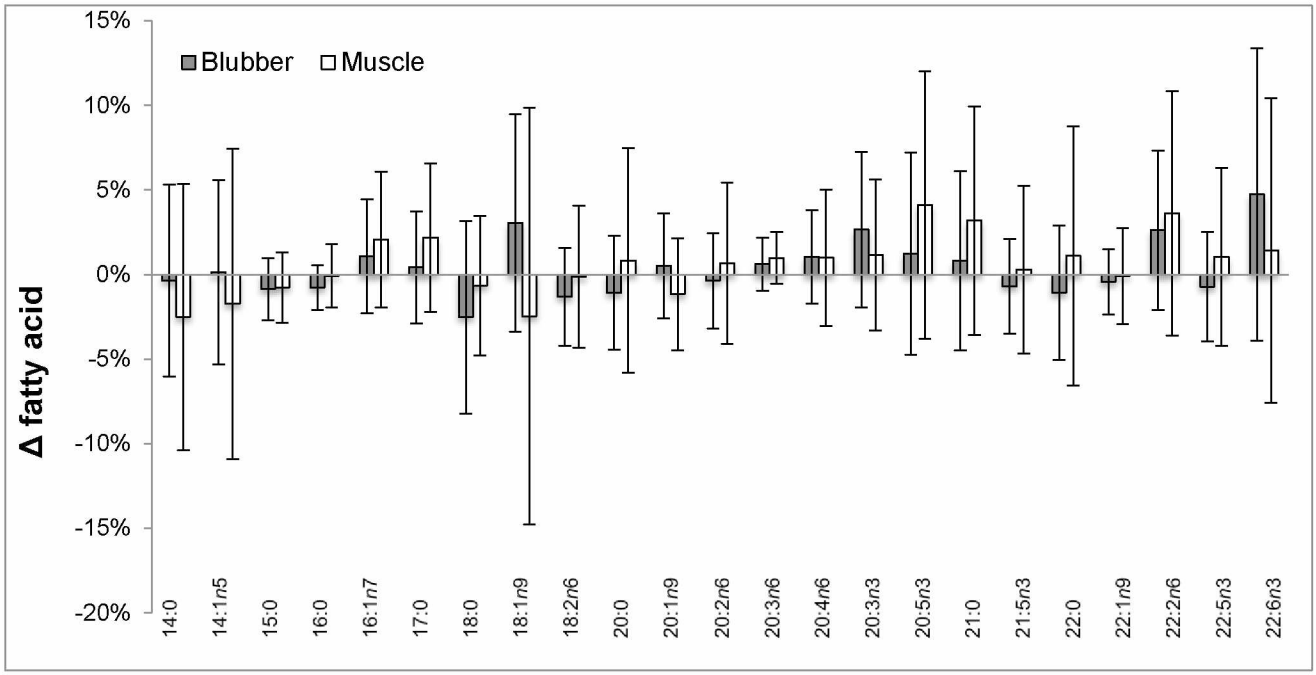

Figure 4.2: Mean ± 1 SD difference in the percentage of fatty acids between fall and spring. Seasonal variation in the relative proportion of individual fatty acids of adult female Weddell seals were handled in both seasons. Samples from each individual were run in the same analytical lab (blubber $n = 11$; muscle $n = 8$). Values > 0% represent a greater relative proportion of a fatty acid present in fall, while values < 0% represent a greater relative proportion of a fatty acid present in spring.

Table 4.1: Mean relative proportions (%) ± 1 SD of 31 fatty acids. Fatty acids were quantified in blubber and muscle of adult female Weddell seals in fall (January) and spring (October) for ASET and Baylor labs.

	Blubber				Muscle			
	ASET		Baylor		ASET		Baylor	
	January	October	January	October	January	October	January	October
Fatty acid	*n* = 15	*n* = 22	*n* = 23	*n* = 9	*n* = 15	*n* = 22	*n* = 23	*n* = 9
12:0	0.16 ± 0.02	0.16 ± 0.03	0.07 ± 0.01	0.07 ± 0.01	0.40 ± 0.10	0.44 ± 0.24	0.23 ± 0.12	0.34 ± 0.19
13:0	0.09 ± 0.02	0.09 ± 0.02	0.05 ± 0.01	0.06 ± 0.00	0.26 ± 0.10	0.24 ± 0.19	0.30 ± 0.33	0.26 ± 0.36
14:0	10.19 ± 0.96	10.85 ± 0.63	8.36 ± 0.92	9.21 ± 0.06	8.50 ± 2.33	9.23 ± 3.73	5.56 ± 2.28	6.36 ± 3.60
15:0	0.41 ± 0.03	0.43 ± 0.04	0.54 ± 0.05	0.59 ± 0.03	0.66 ± 0.13	0.83 ± 0.21	0.87 ± 0.59	1.36 ± 0.63
16:0	9.67 ± 1.22	10.65 ± 1.05	5.32 ± 0.65	5.97 ± 0.49	14.02 ± 2.07	14.38 ± 3.08	13.36 ± 6.77	13.20 ± 6.88
17:0	0.25 ± 0.03	0.24 ± 0.05	0.22 ± 0.05	0.25 ± 0.05	0.73 ± 0.25	0.92 ± 0.43	1.19 ± 1.25	2.10 ± 1.55
18:0	1.90 ± 0.17	1.84 ± 0.22	1.25 ± 0.21	1.40 ± 0.27	7.75 ± 2.41	6.91 ± 2.30	12.34 ± 7.01	12.71 ± 9.52
20:0	0.43 ± 0.12	0.43 ± 0.16	0.11 ± 0.08	0.11 ± 0.13	1.33 ± 0.67	1.89 ± 1.45	0.20 ± 0.11	0.69 ± 1.41
21:0	0.31 ± 0.07	0.31 ± 0.14	0.04 ± 0.04	0.05 ± 0.04	0.87 ± 0.53	0.99 ± 0.74	0.26 ± 0.85	0.14 ± 0.24
22:0	0.51 ± 0.15	0.51 ± 0.21	0.14 ± 0.28	0.15 ± 0.07	1.58 ± 0.90	2.41 ± 1.93	0.46 ± 0.84	0.37 ± 0.61
%ΣSFA	23.92 ± 2.11	25.52 ± 1.81	16.09 ± 1.89	17.86 ± 0.88	36.09 ± 3.02	38.27 ± 4.19	34.78 ± 13.77	37.53 ± 15.97
14:1*n*5	1.88 ± 0.41	1.75 ± 0.42	2.99 ± 0.57	2.57 ± 0.51	1.15 ± 0.19	1.10 ± 0.29	1.45 ± 0.69	1.26 ± 0.74
15:1*n*5	0.16 ± 0.05	0.15 ± 0.06	0.10 ± 0.03	0.07 ± 0.03	0.41 ± 0.20	0.45 ± 0.32	0.23 ± 0.43	0.14 ± 0.24
16:1*n*7	13.96 ± 1.47	14.47 ± 1.19	25.97 ± 2.53	23.87 ± 1.41	8.55 ± 2.06	8.35 ± 3.13	14.08 ± 6.36	14.28 ± 7.59
17:1*n*7	0.42 ± 0.03	0.41 ± 0.05	0.68 ± 0.20	0.65 ± 0.21	0.62 ± 0.25	0.70 ± 0.45	1.11 ± 1.60	0.63 ± 0.49
trans-18:1*n*9	0.79 ± 0.18	0.72 ± 0.18	0.29 ± 0.03	0.29 ± 0.05	1.04 ± 0.19	1.11 ± 0.41	0.17 ± 0.27	0.18 ± 0.20
18:1*n*9	30.15 ± 1.22	29.62 ±1.83	25.72 ± 1.23	24.62 ± 1.85	22.14 ± 4.31	19.51 ± 6.55	15.78 ± 7.16	15.24 ± 8.48
20:1*n*9	4.93 ± 0.67	4.84 ± 0.49	7.42 ± 1.28	7.20 ± 1.23	4.35 ± 0.72	4.00 ± 0.88	5.26 ± 2.87	4.84 ± 2.67
22:1*n*9	1.11 ± 0.23	1.10 ± 0.15	1.49 ± 0.91	1.26 ± 0.28	1.56 ± 0.35	1.83 ± 0.75	2.14 ± 1.94	1.84 ± 1.38
%ΣMUFA	53.41 ± 2.25	53.05 ± 2.81	64.66 ± 2.29	60.54 ± 3.27	39.82 ± 6.02	37.06 ± 9.03	40.22 ± 14.44	38.41 ± 18.07
18:2*n*6	0.81 ± 0.18	0.74 ± 0.31	2.77 ± 0.86	2.79 ± 0.11	1.16 ± 0.54	1.63 ± 1.14	2.35 ± 0.54	1.98 ± 0.61
trans-18:2*n*6	2.06 ± 0.11	1.93 ± 0.31	0.06 ± 0.02	0.07 ± 0.01	1.90 ± 0.34	1.84 ± 0.65	0.32 ± 0.29	0.39 ± 0.42
18:3*n*3	0.36 ± 0.06	0.37 ± 0.13	0.70 ± 0.15	0.65 ± 0.26	0.79 ± 0.39	0.72 ± 0.50	0.49 ± 0.50	0.31 ± 0.33

Table 4.1 continued

	Blubber				Muscle			
	ASET		Baylor		ASET		Baylor	
	January	October	January	October	January	October	January	October
Fatty acid	*n* = 15	*n* = 22	*n* = 23	*n* = 9	*n* = 15	*n* = 22	*n* = 23	*n* = 9
18:3*n*6	0.71 ± 0.06	0.70 ± 0.09	0.28 ± 0.57	0.18 ± 0.02	0.98 ± 0.41	0.87 ± 0.56	0.31 ± 0.60	0.31 ± 0.30
20:2*n*6	0.53 ± 0.09	0.49 ± 0.09	0.30 ± 0.05	0.33 ± 0.05	0.90 ± 0.41	1.24 ± 0.91	0.81 ± 2.19	1.32 ± 2.93
20:3*n*3	4.68 ± 0.06	4.83 ± 1.16	0.36 ± 0.03	0.36 ± 0.07	3.25 ± 0.98	3.17 ± 1.32	1.38 ± 1.31	1.01 ± 1.24
20:3*n*6	0.13 ± 0.03	0.11 ± 0.01	0.19 ± 0.05	0.23 ± 0.08	0.14 ± 0.07	0.09 ± 0.06	2.38 ± 2.90	3.45 ± 2.93
20:4*n*6	0.89 ± 0.10	0.89 ± 0.11	0.09 ± 0.05	0.12 ± 0.04	1.67 ± 0.63	1.73 ± 0.91	1.69 ± 3.44	1.93 ± 3.24
20:5*n*3	0.44 ± 0.14	0.65 ± 1.03	0.48 ± 0.16	0.53 ± 0.07	1.26 ± 0.76	1.47 ± 1.13	2.09 ± 4.17	1.87 ± 3.75
21:5*n*3	0.63 ± 0.11	0.60 ± 0.12	0.28 ± 1.26	0.09 ± 0.04	1.23 ± 0.51	1.48 ± 1.02	1.99 ± 5.00	1.74 ± 4.16
22:2*n*6	0.31 ± 0.08	0.31 ± 0.12	5.46 ± 1.60	6.66 ± 1.81	0.76 ± 0.35	0.91 ± 0.77	4.58 ± 2.80	4.53 ± 3.52
22:5*n*3	0.39 ± 0.11	0.48 ± 0.36	0.97 ± 4.18	0.09 ± 0.04	1.16 ± 0.61	1.37 ± 0.94	1.33 ± 2.40	1.32 ± 2.07
22:6*n*3	8.34 ± 1.61	7.00 ± 1.56	8.18 ± 2.14	9.50 ± 1.85	5.53 ± 2.12	4.23 ± 2.02	5.27 ± 2.11	3.91 ± 1.99
%ΣPUFA	20.27 ± 1.92	19.09 ± 1.50	20.12 ± 3.04	21.60 ± 3.50	20.73 ± 6.03	20.74 ± 7.68	25.00 ± 21.69	24.06 ± 21.36

%ΣSFA: sum total of the relative proportion of all saturated fatty acids; %ΣMUFA: sum total of the relative proportion of all monounsaturated fatty acids; %ΣPUFA: sum total of the relative proportion of all polyunsaturated fatty acids.

Table 4.2: Results of linear models by tissue type. Models show correlations between physiological predictors and the relative proportions of individual fatty acids in blubber and muscle of adult female Weddell seals.

Tissue	Fatty acid	Model	R^2	*p*-value
Blubber	14:0	0.3262 - 0.0290(Lab) + 0.0087(Season)	0.57	> 0.001
n = 69	14:1*n*5	0.0383 + 0.0380(Lab) - 0.0024(Season) + 0.0164(Mass)	0.55	> 0.001
	16:0	0.4234 - 0.08550(Lab) + 0.013(Season) - 0.0179(Mass)	0.87	0.049
	18:1*n*9	0.4562 - 0.0451(Lab) + 0.0206(Mass)	0.69	0.032
	18:2*n*6	0.1424 + 0.0705(Lab) - 0.0016(TBL)	0.69	> 0.001
Muscle	14:1*n*5	0.1509 - 0.1248(TBL)	0.04	0.032
n = 69	15:0	0.0721 + 0.0126(Season)	0.05	> 0.001
	17:0	-0.01815 + 0.0308(Lab) + 0.3063(TBL)	0.12	0.006
	20:0	-0.0855 - 0.0664(Lab) + 0.0353(Mass)	0.50	> 0.001
	20:1*n*9	0.3015 - 0.2729(TBL)	0.05	0.038
	20:3*n*6	-0.1495 + 0.1263(Lab) + 0.5095(TBL)	0.43	> 0.001
	20:4*n*6	-0.0255 + 0.4152(TBL)	0.06	0.021

Input parameters included: Mass (log(mass)), total body lipid (TBL), Reproductive status, Season, and Lab. Where Season is a significant parameter, the coefficient is multiplied by "0" for January, and "1" for October. Where lab is a significant predictor, the coefficient is multiplied by "0" for ASET, and "1" for Baylor. Model selection was based on AICc; only the top model for each fatty acid is presented.

Table 4.3: Results of linear models of seasonal change. Models show the correlations between seasonal changes in the relative proportions of individual fatty acids and physiological predictors in blubber and muscle of adult female Weddell seals handled in both seasons.

Tissue	Fatty acid	Model	R^2	*p*-value
Blubber	14:1*n*5	-0.0145 + 0.0327(Repro status)	0.48	0.011
n = 11	16:1*n*7	0.3091 - 0.1365(Lab) - 0.0007(Jan mass)	0.54	0.019
	17:1*n*7	-0.1340 + 0.0800(Lab) + 0.0003(Jan mass)	0.72	0.003
	18:2*n*6	-0.1838 + 0.0890(Lab) + 0.0005(Jan mass)	0.70	0.003
	18:3*n*3	-0.0502 + 0.0102(TBL change) - 0.0158(Dive resid)	0.69	0.024
	20:0	-0.2741 + 0.1521(Lab) + 0.0006(Jan mass)	0.66	0.005
	20:2*n*6	-0.1878 + 0.1039(Lab) + 0.0005(Jan mass)	0.73	0.002
	20:3*n*3	0.0244 + 0.0254(Dive resid)	0.25	0.119
	20:3*n*6	0.0624 - 0.0340(Lab) - 0.0002(Jan mass)	0.64	0.007
	21:5*n*3	0.0253 - 0.0605(Repro status)	0.35	0.033
	22:0	-0.3162 + 0.1788(Lab) + 0.0007(Jan mass)	0.68	0.004
	22:1*n*9	-0.2542 + 0.0970(Lab) + 0.0006(Jan mass) + 0.0077(TBL change)	0.89	>0.001
	22:2*n*6	-0.0617 + 0.0126(TBL change) - 0.0185(Dive resid)	0.67	0.026
	22:5*n*3	0.0239 - 0.0728(Repro status)	0.29	0.050
Muscle	15:0	0.1723 + 0.0496(Lab) - 0.0005(Jan mass)	0.86	0.003
n = 8	16:0	-0.2820 + 0.0081(Oct TBL)	0.45	0.040
	17:0	0.3439 + 0.0964(Lab) - 0.0009(Jan mass)	0.80	0.007
	18:1*n*9	-0.9017 - 0.3454(Lab) + 0.0025(Jan mass)	0.89	0.002
	trans-18:1*n*9	0.2496 + 0.0641(Lab) - 0.0007(Jan mass)	0.76	0.013
	18:2*n*6	0.3490 + 0.1138(Lab) - 0.001(Jan mass)	0.86	0.003
	trans-18:2*n*6	0.4086 - 0.0053(Oct TBL) - 0.0006(Jan mass)	0.71	0.020
	20:0	0.6553 + 0.1762(Lab) - 0.0018(Jan mass)	0.88	0.002
	20:2*n*6	0.4669 + 0.122(Lab) - 0.0013(Jan mass)	0.81	0.007
	21:5*n*3	0.7938 + 0.1356(Lab) - 0.0015(Jan mass) - 0.0083(Jan TBL)	0.97	> 0.001
	22:0	0.7554 + 0.2035(Lab) - 0.0021(Jan mass)	0.88	0.002
	22:1*n*9	0.2857 + 0.0687(Lab) - 0.0008(Jan mass)	0.75	0.013
	22:5*n*3	0.5695 + 0.1303(Lab) - 0.0015(Jan mass)	0.82	0.006

Input parameters included: log(Jan mass) (Jan mass), January total body lipid (Jan TBL), October total body lipid (Oct TBL), total body lipid change over winter (TBL change), October reproductive status (Repro status), dive residual (Dive resid), and Lab. Where Reproductive status is a significant parameter, the coefficient is multiplied by "0" for non-reproductive, and "1" for reproductive. Where Lab is a significant predictor, the coefficient is multiplied by "0" for ASET, and "1" for Baylor. Linear models were run for all fatty acids detected above trace (≥ 0.5%), only fatty acids with significant predictors are presented, and models that only included Lab are not reported. Model selection was based on AICc; only the top model for each fatty acid is presented.

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General Conclusions

Blubber is central to thermoregulation of adult phocids. For pups that lack blubber, lanugo provides equivalent insulation, and neonates of harp, hooded, and Weddell seals in the present study had similar overall insulation. However, the lack of blubber in some species is associated with small body size, which creates a high surface area to volume ratio (SA:V), increasing the potential for heat loss. Further, lanugo is an ineffective insulation in water or when wet. For species with a high potential for water immersion, pups may need heat generating mechanisms to rewarm the body if immersion occurs. The primary heat generating mechanism varies among species, with species having greater capacity for either nonshivering thermogenesis (NST) or shivering thermogenesis (ST). The present study was the first to confirm that harp seals express UCP1, and should thus be capable of NST. Grav et al. (1974) proposed the importance of NST in young harp seals, but the expression of UCP1 had not been confirmed. The prevalence of uncoupling protein 1 (UCP1), and the potential for NST among other phocid species is largely unknown (Sakurai et al. 2015). Further, the use and reliance on NST, and the energetic consequences, have not been measured in any phocid species.

Pups must develop the thermoregulatory capabilities of 'aquatic' adults from a 'terrestrial' starting point in a relatively short period (days to weeks), because prolonged immersion during early independent foraging without the physiological capabilities to defend against higher rates of heat loss would energetically compromise young animals (Liwanag et al., 2009). For species born with lanugo and in an environment with a high potential for immersion, such as harp, spotted (*Phoca largha*), and ringed seals (*P. hispida*), NST or ST is likely essential for maintaining euthermia and drying the coat (Blix and Steen, 1979; Smith et al., 1991). However, NST and ST come at a high metabolic cost (Cannon and Nedergaard, 2004; de Meis et al., 2005), reducing the energy available for growth and development. Heavy reliance on such thermogenic mechanisms as NST or ST could slow growth rates and potentially lower juvenile survival. Investigating the expression of UCP1, NST capability, and regulation in other ice dependent phocid pups will help us understand the energetic consequences of these thermogenic mechanisms. For example, researching the thermoregulatory mechanisms used by developing Weddell seals, the metabolic costs, and the impacts on development and juvenile survival are needed to understand how the southernmost mammal transitions to independence.

Harp seals, like other phocids, are dependent on a stable sea ice substrate during the nursing and postweaning fast periods (Bajzak et al., 2011; Friedlaender et al., 2010; Moore and Huntington, 2008). Current environmental conditions in the Arctic are warming rapidly, resulting in reduced sea ice stability, depth, duration (Friedlaender et al., 2010; Hansen et al., 2013), and an increase in rain and storm events (Schultz, 2013; Vermaire et al., 2013). Poor ice conditions are known to increase pup mortality (Bajzak et al., 2011; Ferguson et al., 2005; Friedlaender et al., 2010; Kovacs and Lydersen, 2008). Our results suggest if nursing harp seal pups are forced to enter the water early as a result of poor ice conditions, they would have elevated thermoregulatory costs as compared to weaned pups, because of their lack of blubber. The increased thermoregulatory costs could have negative consequences on their survival (Davydov and Makarova, 1964; Smith and Harwood, 2001; Worthy, 1991). While harp seals gain blubber quickly (Kovacs and Lavigne, 1985), and thus have a relatively short period of vulnerability, this may not be the case in species with prolonged development periods and smaller birth size, such as ringed and spotted seals (Ferguson et al., 2005; Oftedal et al., 1996). As pups of seven of the eleven phocid species associated with sea ice at either poles rely on lanugo when young, these findings suggest that vulnerability to changes in ice conditions or rain events may be widespread. While it may be possible for some species to shift from pupping on pack ice to nearby shorelines, such changes pose other risks such as increased predation, disturbance, and disease transmission (Bajzak et al. 2011; Friedlaender et al. 2010). Ultimately, more research is needed to understand the mechanisms, metabolic costs, and timing associated with development of thermoregulatory strategies in young polar marine mammals as they likely vary by species with different early thermoregulatory strategies. This would create better predictions of the response to climate change of different ice-breeding seals during the critical time of early development (Huey et al., 2012).

Blubber was the most predominant way the challenges and costs of aquatic living were met by phocids in this study. However, the importance of physiological/ecological drivers on lipid composition of blubber, or environmental influence on blubber apart from whole-animal thermoregulation, was not well understood. Findings of the present study indicate blubber is not merely a static storage site, but instead a highly regulated and dynamic tissue, the composition of which reflects environment, thermodynamics, diet, and body size. Among phocid adults, there are clear correlations between latitude (as a proxy for environmental temperature) and the

relative proportions of FA classes of the blubber. This pattern was evident despite large variation in diet, blubber thickness, and lipid content of blubber among species. This suggests environmental interaction between blubber lipids and temperature results in modulation of the FA in the blubber to deal with different environmental conditions. Latitude appears to have a greater influence on blubber composition than phylogeny. Closely related species, and population among the same species, followed the trends with latitude rather than grouping by tribe or species. Because the most superficial, or outer layer of blubber typically contains the steepest temperature gradient (Strandberg et al., 2011, 2008), the FA composition of this layer may be the most strongly influenced by environment. The influence of environmental temperature among species on the FA composition of specifically this layer may be stronger than the relationship with the whole thickness of the blubber. Further, the FA composition of the inner layers of blubber may have weaker relationships with environment, as the inner blubber layer is more metabolically active and kept at warmer temperatures (Strandberg et al., 2011).

There are clear differences in the FA composition of blubber and muscle, of harp, hooded, and Weddell seals, and body mass and condition appear to influence the FA in these tissues in opposite ways. The influence of mass and blubber depth on the relative proportions of FA classes in the muscle among species is likely related to the influence both mass and body condition have on metabolic rate (Aarseth et al., 1999; Kleiber, 1975; Nagy, 2005; Weibel and Hoppeler, 2005). Similarly, muscle lipid stores are also highly regulated, with variation in FA profile attributable to animal size and body condition. We did not have a large enough sample size to test the relationships between latitude and individual fatty acids. Few individual FA within a class may contribute to a tradeoff between fluidity and thermoregulation for an individual. The difference in the FA composition of blubber and muscle of adult female Weddell seals was driven by four individual fatty acids, which made up ~50% of the total FA in each tissue. These FA were also influenced by physiological parameters, such as body mass and body condition.

The differences in FA class profiles between muscle and blubber likely reflect blubber's primary function as insulation as MUFA remain fluid at lower temperatures. In contrast, SFA are fluid at higher temperatures, and are dense sources of energy for muscle. Body size and total body lipid directly affect thermoregulation; thus, it follows that this would be an important predictor of the FA composition of the blubber layer. Though it has been suggested by previous

studies (Fahlman, 2012; Trumble and Kanatous, 2012; Trumble et al., 2010), we did not find any clear relationship between dive duration on the FA profiles of muscle or blubber. There may be other factors while diving (i.e., diet, fat calorie intake) that may account for the different patterns of FA allocation and seasonal effects between small and large animals. We need to understand if FA classes or individual FA influence the conductivity of blubber, which directly impacts thermoregulation. Metabolic tracer studies are needed to identify the sources of FA that are deposited in blubber and muscle, and to determine if particular classes or individual FA are used preferentially in skeletal muscle during diving. Tracer studies could also determine if the pattern of mobilization of FA from the blubber to muscle, and within the muscle, are similar to those of terrestrial animals (Raclot, 2003) or are unique, as in other species with lipid-based metabolism (Price et al., 2013). This would shed light on the importance of specific FA fuel sources in the muscle while diving. If particular classes or individual FA are disproportionately metabolized, potential limitations on performance may occur if the dietary source of these FA (or their precursors) changes in availability or distribution in response to fishing (Ainley et al., 2013) or climate change (Ainley et al., 2013; Mintenbeck et al., 2012; Smith et al., 2007).

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Appendix A.

Coauthor approval of manuscript inclusion in the dissertation.

Permission of Co-Authored Manuscript Inclusion in UAF Dissertation

As co-author on the manuscripts entitled, "To each their own: thermoregulatory strategy varies among neonatal polar phocids" published in Comparative Physiology and Biochemistry A, and, "Shifts in thermoregulatory strategy during ontogeny in harp seals (*Pagophilus groenlandicus*)" published in the Journal of Thermal Biology, I give permission for Linnea Pearson to include these papers as chapters in her graduate student dissertation to the University of Alaska Fairbanks.

Citations:

Pearson LE, Liwanag HEM, Hammill MO, Burns JM. To each it's own: thermoregulatory strategy varies among neonatal polar phocids. Comparative Biochemistry and Physiology Part A 138: 59-67. (doi: 10.1016/j.cbpa.2014.08.006).

Pearson LE, Liwanag HEM, Hammill MO, Burns JM. 2014. Shifts in thermoregulatory strategy during ontogeny in harp seals (*Pagophilus groenlandicus*). Journal of Thermal Biology (doi: 10.1016/j.thermbio.2014.02.001).

Co-Author:

Signature: Hammill, Mike Digitally signed by Hammill.Mike DN: c=CA, o=GC, ou=DFO-MPO, cn=Hammill.Mike Date: 2015.06.17 07:34:16 -04'00' Date: _____

Permission of Co-Authored Manuscript Inclusion in UAF Dissertation

As co-author on the manuscripts entitled, "To each their own: thermoregulatory strategy varies among neonatal polar phocids" published in Comparative Physiology and Biochemistry A, and, "Shifts in thermoregulatory strategy during ontogeny in harp seals (*Pagophilus groenlandicus*)" published in the Journal of Thermal Biology, I give permission for Linnea Pearson to include these papers as chapters in her graduate student dissertation to the University of Alaska Fairbanks.

Citations:

Pearson LE, Liwanag HEM, Hammill MO, Burns JM. To each it's own: thermoregulatory strategy varies among neonatal polar phocids. Comparative Biochemistry and Physiology Part A 138: 59-67. (doi: 10.1016/j.cbpa.2014.08.006).

Pearson LE, Liwanag HEM, Hammill MO, Burns JM. 2014. Shifts in thermoregulatory strategy during ontogeny in harp seals (*Pagophilus groenlandicus*). Journal of Thermal Biology (doi: 10.1016/j.thermbio.2014.02.001).

Co-Author:

Signature: Heather Liwanag Date: 6/15/15

Appendix B.

Permit and IACUC permissions.

University of Alaska Anchorage
Institutional Animal Care and Use Committee

Memo

To: Dr. Jennifer Burns

From: UAA IACUC

Date: 3/1/2005

Re: IACUC protocol # 2005Burns1

Dr. Burns –

The Institutional Animal Care and Use Committee at the University of Alaska Anchorage has approved your protocols "Heme and Muscle Development in Hooded and Harp Seals", pending receipt of required state permits. Your approval is good for a period of 3 years, and will expire March 1, 2008. You are required to submit an annual report of your activities (form available at http://www.uaa.alaska.edu/iacuc/protocols.html) by March 1st of each year. We would also like to remind you that any changes in personnel or protocols must also be submitted to the committee. Thank you for your support of animal care guidelines. We hope that your research goes well.

Dr. E. Murphey for UAA IACUC

UAA UNIVERSITY of ALASKA ANCHORAGE

University of Alaska Anchorage
Institutional Animal Care and Use Committee

Memo

To: Dr. Jennifer Burns

From: Eric S. Murphy, Chair, IACUC

Date: 11/19/2009

Re: IACUC protocol #2009Burns1

A designated reviewer, appointed by the Institutional Animal Care and Use Committee at the University of Alaska Anchorage, reviewed your protocol "Collaborative Research: Weddell Seals as Autonomous Sensors of the Winter Oceanography of the Ross Sea (PI: D.P Costa, UC Santa Cruz)," that you submitted to us on 10/22/2009. Upon receiving further information from you on 11/18/2009, I am pleased to approve your protocol. Please note that this approval is contingent upon your compliance with all relevant University, city, state, and federal regulations, and requires that you possess all relevant permits before work is initiated. Your protocol ID number is **2009Burns1**. Your approval is good for a period of 3 years from when the protocol was originally approved by the University of Santa Cruz's IACUC, and will expire on **7/2/2012**. You are required to submit an annual report of your activities prior to **7/2** in each of the next two years. This form is available at http://www.uaa.alaska.edu/research/ric/iacuc/forms.cfm .

We remind you that all changes in personnel and animal handling protocols must be submitted to the committee prior to such changes taking place. In addition, should you experience any unexpected animal mortalities, illnesses, or injury (to animals or personnel involved with the project), you are required to report such to the IACUC immediately.

Thank you for your support of animal care guidelines. We hope that your research goes well.

Eric S. Murphy

Eric S. Murphy, Chair
UAA IACUC

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DATE:	June 14, 2011
TO:	Jennifer Burns, PhD
FROM:	University of Alaska Anchorage IACUC
STUDY TITLE:	Weddell seals at Autonomous sensors of the winter oceanography of the Ross Sea
IRB REFERENCE #:	[177250-2]
SUBMISSION TYPE:	Continuing Review/Progress Report
ACTION:	APPROVED
APPROVAL DATE:	June 10, 2011
EXPIRATION DATE:	July 2, 2012
REVIEW TYPE:	Full Committee Review

The annual renewal for your UAA IACUC project [177250-2] *Weddell seals at Autonomous sensors of the winter oceanography of the Ross Sea* was approved on June 10, 2011. You are, therefore, authorized to continue research on vertebrate animals as authorized under this protocol. Your next renewal will be due on July 2, 2012 and the project approval expires on **July 2, 2012**.

Please submit the pathology report on Seal 11-02 when it is available.

We remind you that all changes in personnel and animal handling protocols must be submitted to the committee prior to these changes taking place. In addition, should you experience any unexpected animal mortalities, illnesses, or injury (to animals or personnel involved with the project), you are required to report such to the IACUC immediately.

Thank you for your support of animal care guidelines.

Eric S. Murphy

Eric S. Murphy, Ph.D.

Chair, UAA IACUC

UNITED STATES DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
NATIONAL MARINE FISHERIES SERVICE

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February 15, 2008 F/AKC4: rpa

Dr. Jennifer Burns
Department of Biological Sciences, EBL 123
University of Alaska Anchorage
3211 Providence Drive
Anchorage, AK 99508

Dear Dr. Burns: Jenn

You and your graduate students, Keri Lestyk-Lodge and Linnea Pearson, are authorized as a Co-Investigators (CI), to conduct research under the NMML Permit #782-1694 issued pursuant to the Marine Mammal Protection Act (MMPA). You are specifically authorized to import, export and possess

- Hooded seal muscle, blood, blubber, whiskers, skin, and organs collected in Canada;
- Harp seal muscle, blood, blubber, whiskers, skin, and organs collected in Canada

This letter announces the extension of this authorization until **31 December 2008** and serves as a reminder of your responsibilities under this permit. As a CI working under a NMML permit, you are reminded that:

1) You are responsible for reading, understanding, and fully complying with all provisions of the research permit under which you are authorized;

2) You must comply with NMFS Office of Protected Resources policy that prohibits the use of research-associated photography for commercial purposes without prior approval from the Office of Protected Resources (see http://www.nmfs.noaa.gov/pr/permits/faq_mmpermits.htm for details);

3) As the CI, you are the on-site representative of NMML under this permit. No person who is not specifically listed as a CI on the permit may import, export, or possess specimens in the absence of the NMML staff to whom the permit is issued (see http://www.nmfs.noaa.gov/pr/permits/faq_mmpermits.htm for details).

4) As a condition of being authorized to conduct research under a NMML permit, you are required to provide NMML with both annual and final reports of your activities. Reporting requirements for Permit #782-1694 can be found in Section C of the permit. An annual report of your activities must be received by NMML no later than **1 March 2008**, and your

UNITED STATES DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
NATIONAL MARINE FISHERIES SERVICE
Silver Spring, MD 20910

NOV 0 4 2011

Daniel P. Costa, Ph.D.
Long Marine Laboratory
University of California at Santa Cruz
100 Shaffer Road
Santa Cruz, California 95060

Dear Dr. Costa:

The National Marine Fisheries Service has issued Permit No. 87-1851-04, which amends and replaces Permit No. 87-1851-03, for research activities on marine mammals. The changes to specific Terms and Conditions are reflected in bold font. This permit is effective upon your signature and valid through the expiration date of December 31, 2012.

You must return the "file copy" signature page, with your dated signature, to this office as proof of your acceptance of the permit. There are two signature pages in the enclosed permit. Please sign and date both pages. Keep the original signature page with the rest of the permit as proof of your authorization to conduct the research activities.

Please read your permit carefully before signing it. If you have questions, please contact your permit analyst – Amy Sloan or Tammy Adams – at 301-427-8401 before signing the permit. If you need assistance with your permit in the future, please contact one of these permit analysts.

Please return the signature page marked "file copy" to the Chief, Permits Division (F/PR1), 1315 East-West Highway, Silver Spring, MD 20910. You may also submit the "file copy" of the signature page by email (Amy.Sloan@noaa.gov) or fax (301-713-0376) and confirm it by mail.

Sincerely,

P. Michael Payne
Chief, Permits, Conservation
and Education Division
Office of Protected Resources
(phone: 301-427-8401)

Enclosure

UNITED STATES DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
NATIONAL MARINE FISHERIES SERVICE
Silver Spring, MD 20910

APR 2 5 2011

Jennifer Burns, Ph.D.
University of Alaska Anchorage
CPISB 202C
3101 Science Circle
Anchorage, AK 99508

Dear Dr. Burns:

The National Marine Fisheries Service has issued Permit No. 15510 to you for research activities on marine mammal parts. This permit is effective upon your signature and valid through the expiration date indicated in Condition A.1.

Here's what you need to do to use your permit:

1. Read the permit, including attachments. If you have questions, call your permit analyst – Amy Sloan or Laura Morse – at 301-713-2289 before signing the permit.
2. Sign and date the original signature page and the signature page marked "file copy."
3. Keep the original signature page with your permit. You need both as proof of your authorization to conduct the research activities.
4. Send the "file copy" signature page to our office as proof of your acceptance of the permit.

Please keep your email contact information current in our online database (https://apps.nmfs.noaa.gov/). You will receive automated email reminders of due dates for annual and final reports, and a notice prior to expiration of your permit.

Please return the signature page marked "file copy" to the Permits Division (F/PR1), 1315 East-West Highway, Silver Spring, MD 20910. You may also submit the "file copy" of the signature page by facsimile (FAX number: 301-713-0376) and confirm it by mail.

Sincerely,

P. Michael Payne
Chief, Permits, Conservation
and Education Division
Office of Protected Resources
(phone: 301-713-2289)

Enclosure